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BIOLOGICAL BULLETIN

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B

CONTENTS

No. 1. February, 1955 PAGE

Ball, Eric G., Cornelius F. Strittmatter and Octavia Cooper
Metabolic studies on the gas gland of the swim bladder..... 1

Bonner, John Tyler
A note concerning the distribution of polysaccharides in the early development of the hydromedusan Phialidium gregarium..... 18

Green, James W., and Jay S. Roth
The effect of radiation from small amounts of P^{32}, S^{35} and K^{42} on the development of Arbacia eggs..... 21

Gregg, John R., and Søren Løvtrup
Synthesis of desoxyribonucleic acid in lethal amphibian hybrids..... 29

Hamer, D.
The composition of the basic proteins of echinoderm sperm..... 35

Johansson, Arne Semb
The relationship between corpora allata and reproductive organs in starved female Leucophaea maderae (Blattaria)..... 40

McShan, W. H., Sol Kramer and N. F. Olson
Adenosinetriphosphatase activity of American cockroach and woodroach thoracic muscle..... 45

McWhinnie, Mary A.
The effect of colchicine on reconstitutional development in Dugesia dorotocephala..... 54

Smyth, Thomas, Jr., and Chester C. Roys
Chemoreception in insects and the action of DDT..... 66

Spoor, W. A.
Loss and gain of heat-tolerance by the crayfish..... 77

Travis, Dorothy F.
The molting cycle of the spiny lobster, Panulirus argus Latreille. II. Preecdysial histological and histochemical changes in the hepatopancreas and integumental tissues..... 88

de Villafranca, George W.
Adenosinetriphosphatase activity of squid mantle muscle (Loligo pealii).. 113

No. 2. April, 1955

Andrews, E. A.
Some minute movements in protoplasm..... 121

HARGIS, WILLIAM J., JR.
Monogenetic trematodes of Gulf of Mexico fishes. Part I. The superfamily Gyrodactyloidea 125

LEHMAN, H. E.
On the development of enucleated Triton eggs with an injected blastula nucleus 138

LOOSANOFF, V. L., AND C. A. NOMEJKO
Growth of oysters with damaged shell-edges 151

MCWHINNIE, M. A., AND H. M. SWEENEY
The demonstration of two chromatophorotropically active substances in the land isopod, Trachelipus rathkei 160

MOORE, HILARY B.
Variations in temperature and light response within a plankton population. 175

POWERS, E. L., C. F. EHRET AND L. E. ROTH
Mitochondrial structure in Paramecium as revealed by electron microscopy 182

RAFFERTY, KEEN A., JR.
The fates of segments from Tubularia primordia 196

RICHARDS, A. GLENN, AND MYRTLE N. SMITH
Infection of cockroaches with Herpomyces (Laboulbeniales). I. Life history studies 206

STEINBERG, MALCOLM S.
Cell movement, rate of regeneration, and the axial gradient in Tubularia.. 219

STEPHENS, GROVER C.
Introduction of molting in the crayfish, Cambarus, by modification of daily photoperiod 235

TRUONG, SIMONE THI-HUE, AND ERNST J. DORNFELD
Desoxyribose nucleic acid content in the nuclei of salamander somatic tissues 242

No. 3. JUNE, 1955

CLARK, D. P.
The influence of body weight, temperature and season upon the rate of oxygen consumption of the terrestrial amphipod, Talitrus sylvaticus (Haswell) 253

CLEMENTS, MARGARET, AND AUBREY GORBMAN
Protease in ammocoetes endostyle 258

GRIFFIN, DONALD R., AND TIMOTHY H. GOLDSMITH
Initial flight directions of homing birds 264

GROSCH, DANIEL S., AND HOWARD E. ERDMAN
X-ray effects on adult Artemia 277

KOZLOFF, EUGENE N.
Lwoffia cilifera Gen. Nov., Sp. Nov., a ciliated member of the family Sphenophryidae (Holotricha: Thigmotricha) 283

LAUFFER, MAX A., AND LOUIS G. SWABY
The size, shape and hydration of lobster hemocyanin..... 290

NAYAR, K. K.
Studies on the neurosecretory system of Iphita limbata Stal. I. Distribution and structure of the neurosecretory cells of the nerve ring..... 296

ROTH, JAY S., AND HERBERT J. EICHEL
The effect of x-radiation on enzyme systems of Tetrahymena pyriformis.. 308

RUGH, ROBERTS, AND HELEN CLUGSTON
Effects of various levels of x-irradiation on the gametes and early embryos of Fundulus heteroclitus..... 318

SMITH, RALPH I.
On the distribution of Nereis diversicolor in relation to salinity in the vicinity of Tvärminne, Finland, and the Isefjord, Denmark..... 326

JODREY, LOUISE H., AND KARL M. WILBUR
Studies on shell formation. IV. The respiratory metabolism of the oyster mantle..... 346

WILBUR, KARL M., AND LOUISE H. JODREY
Studies on shell formation. V. The inhibition of shell formation by carbonic anhydrase inhibitors..... 359

ZEUTHEN, ERIK
Mitotic respiratory rhythms in single eggs of Psammechinus miliaris and of Ciona intestinalis..... 366

PR

Volume 108 Number 1

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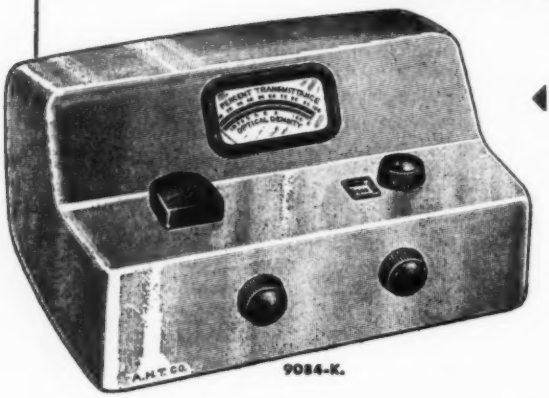

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METABOLIC STUDIES ON THE GAS GLAND OF THE SWIM BLADDER [1]

ERIC G. BALL, CORNELIUS F. STRITTMATTER [2] AND OCTAVIA COOPER

The Marine Biological Laboratory, Woods Hole and the Department of Biological Chemistry, Harvard Medical School, Boston 15, Mass.

The main function of the swim bladder of teleosts is assumed to be that of a hydrostatic organ. It is filled with a mixture of oxygen, carbon dioxide and nitrogen, so that the bladder is normally inflated to a volume which in marine forms is approximately 5% of the total body volume (Jones and Marshall, 1953). This serves to bring the specific gravity of the fish close to that of the sea water in which it lives. The composition of the swim bladder gases varies with the depth of water at which the fish dwells. As the hydrostatic pressure due to depth increases, most species keep their bladder volume constant by increasing mainly its oxygen content. Thus one finds at depths where the hydrostatic pressure is equivalent to 100 atmospheres or so that the percentage of oxygen in the swim bladder gases reaches values well over 90% (Scholander and van Dam, 1953). In such cases this means, in round figures, that a volume of oxygen (as measured at one atmosphere of pressure) equal to 100 times the volume of the swim bladder has been introduced. This amazing feat of introducing oxygen into the swim bladder in relatively large amounts and against extremely high pressures is attributed to the functioning of the so-called gas gland of the swim bladder. The structure and function of this gland has been adequately described in two recent reviews (Fänge, 1953; Jones and Marshall, 1953) and needs no further description here. The mechanism as to how this is achieved by the gas gland remains unproven. At the time the work to be described here was begun in the summer of 1950, little attention had been paid to the metabolic activity of this gland. In view of the concept that acidification of the blood passing through this gas gland might furnish a mechanism for the release of oxygen and carbon dioxide from the blood, it seemed worthwhile to obtain some quantitative data on the gland's oxygen consumption and glucose utilization. It will be shown here that the gas gland possesses a very high capacity to convert glucose to lactic acid and the possible implications of this acid formation on the mechanism of gas production will be discussed. A preliminary report of a portion of this work has been published (Strittmatter, Ball and Cooper, 1952).

[1] This work was supported in part by funds received from the Eugene Higgins Trust through Harvard University.

[2] Lalor Foundation Fellow, Summer, 1952.

METHODS

The fish used in this investigation were generally freshly caught. It was found that fish held in an aquarium for any length of time gave less reproducible results. They were killed by severing the head. The abdomen was opened, the swim bladder exposed and the ventral portion with the gas gland attached was excised. The gas gland, including the *rete mirabile*, was then easily stripped from the bladder wall, it being necessary only to cut the blood vessels at the point where they entered the swim bladder wall. In the scup this yielded a compact but thin piece of intact tissue of ideal dimensions for metabolic studies. On the average (100 fish) we have obtained 100 mg. of gland per each 188 g. of fish. No attempt has been made at further dissection of this piece of tissue and throughout it will be referred to for convenience as the gas gland though it should be pointed out that we are mindful of the fact that a considerable portion of this

TABLE I

Oxygen consumption of the gas gland and several other tissues of fish. Temp. 29.45° C.

Species	Tissue	No. of expts.	Average O_2 consumption
			μl./100 mg./hr.
Scup	Gas gland	10	29.3
(*Stenotomus chrysops*)	Red blood cells	1	8.0*
	Heart slices	1	32.0
	Swim bladder wall	1	3.0
Sea Robin (*Prionotus carolinus*)	Gas gland	1	22.0
Puffer	Gas gland	2	28.0
(*Spheroides maculatus*)	Swim bladder wall	1	2.0
	Red blood cells	1	5.0*

* Values per 0.1 ml. of cells packed by centrifugation. In all cases the suspending medium was 2.8 ml. of the NaCl-phosphate mixture described under Methods and no substrate was added.

piece of tissue is not glandular in nature. The tissue was weighed immediately on a Roller-Smith balance and usually immersed directly in a buffered salt mixture contained in a manometric vessel.

Two general types of buffered media were employed. For the measurement of oxygen consumption we have employed a phosphate-saline mixture with a composition such that the final concentration of phosphate was 0.02 *M* and the total concentration of ions such that it was isotonic with 0.25 *M* NaCl. This was achieved by mixing 20 ml. of 0.1 *M* phosphate buffer, pH 7.4, with 44.4 ml. of 0.5 *M* NaCl and diluting to a volume of 100 ml. The pH of this mixture as measured with the glass electrode at 30° C. was 7.37. Unless otherwise stated, we have employed a medium 0.025 *M* in $NaHCO_3$ and 0.225 *M* in NaCl for the measurement of glycolysis rates.

The conventional Warburg manometric procedure was employed for measurement of oxygen consumption with air as the gas phase and 0.2 ml. of 20% KOH

in the center well. Anaerobic glycolysis rates were measured in the same apparatus by following the release of CO_2 from bicarbonate with a gas mixture of 5% CO_2 and 95% N_2. When aerobic glycolysis rates were measured the gas phase was 5% CO_2 and 95% O_2. The volume of media employed in all glycolysis experiments was 3.0 ml.

Glucose was determined by the method of Nelson as modified by Somogyi (1945) and lactic acid by the method of Barker and Summerson (1941).

Results

Oxygen consumption

The results of measurements of the oxygen consumption of the intact gas gland and of several other tissues from three species of fish are given in Table I. The data are expressed in terms of 100 mg. of wet tissue to facilitate comparison, though as much as five times this amount of tissue may have been actually used in the determination. Little difference was to be noted in the activity of the gas gland from the three different species. Most of the data were obtained on

TABLE II

Dry weight of the gas gland of the scup (Stenotomus chrysops)

Wet weight mg.	Dry weight mg.	%
135.5	19.8	14.6
158.5	26.4	16.6
105.0	15.6	14.8
125.0	18.7	14.9
	Average	15.2

scup which was used almost exclusively throughout this entire investigation since this fish was not only plentiful, but possessed a gas gland which had a less racemose structure than some other species investigated. In view of the highly vascular nature of the gland the oxygen consumption of the nucleated red blood cells was measured. The data show that the oxygen consumption of the gland is due chiefly to the tissue itself and not to the red blood cells contained in it. The wall of the swim bladder, as is to be expected from its histological structure, has a low oxygen consumption. One determination on the oxygen consumption of heart muscle slices yielded values comparable to that of the gas gland.

The dry weight of the gas gland from the scup was determined by drying the tissue in an oven at 100° C. for 3–4 hours and then placing in a desiccator over $CaCl_2$ overnight before weighing. The data given in Table II indicate that the average dry weight of the gas gland may be taken in round figures as 15%. On this basis a Q_{O_2} calculated for the gas gland of scup from the data given in Table I is approximately 2 μl. per hour per mg. dry weight.

The oxygen consumption of the intact gas gland was optimal when suspended in a medium containing predominantly Na^+ as the cation. Media prepared with KCl in place of NaCl or mixtures of KCl and NaCl gave lower Q_{O_2} values. Mix-

tures of equal parts of NaCl and sea water diluted so as to be isotonic gave still lower values.

The values given in Table I are all without added substrate. The addition to the flasks of 0.2 ml. of isotonic glucose (0.5 *M*) did not increase the rate of O_2 uptake (as shown in Curve 1, Fig. 1) and sometimes depressed it. This latter may be the result of the rapid lactic acid production from glucose which occurs, as data to be presented below will show.

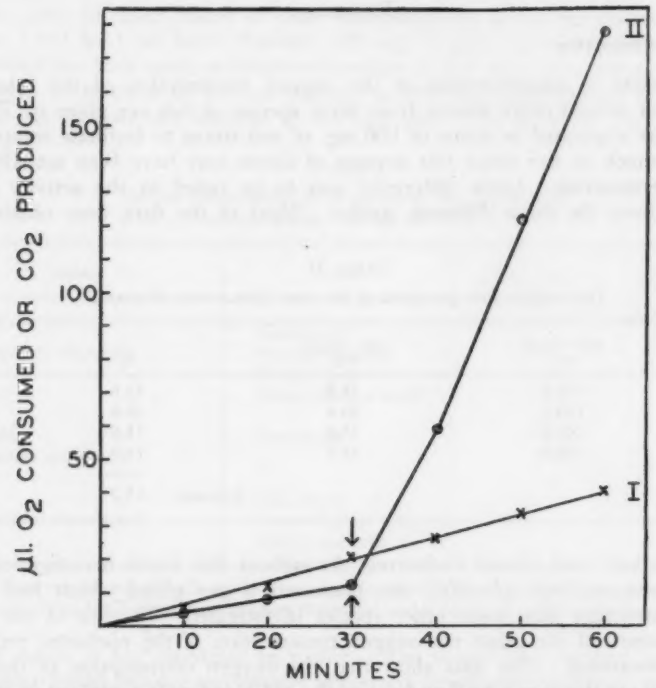

FIGURE 1. Oxygen consumption and anaerobic glycolysis of the gas gland. Curve 1; Oxygen consumption, tissue weight 189 mg. Curve 2; Glycolysis as measured by CO_2 evolution, tissue weight 122 mg. In both cases glucose was added at the point indicated by the arrow so as to yield a final concentration of 300 mg. per 100 ml. Temp. 29.45° C. Media as described under Methods.

Homogenates of the gas gland were prepared with the aid of a glass homogenizer of the Potter-Elvehjem type (Potter and Elvehjem, 1936) in cold NaCl-phosphate medium. The oxygen consumption of such homogenates without added substrate ranged from 2–6 μl. of O_2 per 100 mg. of wet tissue. The addition of succinate or para-phenylene diamine brought the oxygen consumption values of these homogenates up to the range found for the intact gland without added substrate. If cytochrome *c* was added as well, a further small increase in O_2 uptake

was observed. Addition of glucose to gland homogenates caused no significant alteration in oxygen consumption. The addition of sodium hexose diphosphate did stimulate respiration but to only half the extent that succinate did. It was observed that flasks to which hexose diphosphate had been added were strongly acid to phenol red at the end of the run. This suggested that glycolysis was occurring and turned our attention to this aspect of the problem.

Glycolysis

The conversion of glucose to lactic acid by the gland is most conveniently followed manometrically by following the release of CO_2 from bicarbonate. In

TABLE III

Anaerobic glycolysis

	Experiment I				Experiment II			
	No glucose added		Glucose added to give 75 mg./100 ml.		No glucose added		Glucose added to give 150 mg./100 ml.	
Vessel	1	2	3	4	1	2	3	4
Gland wt., mg.	70	74	111	106	85	141	69	82
Initial glucose, μmols	0.20	—	13.21	—	0.67	—	25.06	—
Initial lactate, μmols	0.40	—	0.70	—	1.03	—	1.07	—
Final glucose, μmols	—	0.13	—	8.17	—	0.67	—	21.56
Final lactate, μmols	—	0.66	—	7.64	—	1.75	—	7.41
CO_2 evolved, μmols	—	0.57	—	7.57	—	0.61	—	6.59
Total glucose consumed, μmols	0.07		5.04		0.00		3.50	
Total lactic acid produced, μmols	0.26		6.94		0.72		6.34	
CO_2 release accounted for by lactic acid production	46%		92%		118%		96%	
Glucose consumption accounted for by lactic acid production	186%		69%		—		91%	

Gas phase 5% CO_2–95% N_2. Temp. 30.1° C. Total fluid volume 3.0 ml.

Figure 1, Curve 2, data are plotted for a typical experiment. There is a fourteen-fold increase in the rate of CO_2 production after tipping glucose into the medium. The data given in Table III clearly show that CO_2 production in such an experiment is due to the conversion of glucose to lactic acid. In each of these experiments two pairs of vessels were set up, each containing an intact gas gland. Usually an effort was made to have approximately equal amounts of tissue in each vessel of a pair. One pair of vessels contained glucose in the side arm. All vessels were gassed with 5% CO_2—95% N_2 and equilibrated in the constant temperature bath for five minutes. The vessels containing glucose were tipped and immediately aliquots of the contents of one flask of each pair were removed for

glucose and lactic acid analysis. The other two vessels remained in the bath for one hour during which CO_2 production was measured. At the end of the hour the contents of each of these flasks were immediately prepared for glucose and lactic acid measurements. Total glucose consumption and lactic acid production were then obtained by the difference in values of the initial and final measurements for each set of vessels. It can be seen from the calculations presented in Table III that in each of the two experiments where glucose was added lactic acid production accounted for 92 and 96% of the CO_2 released and 70 and 90% of the glucose that disappeared was accounted for by lactic acid. (It is assumed that each glucose molecule yields two of lactic acid.)

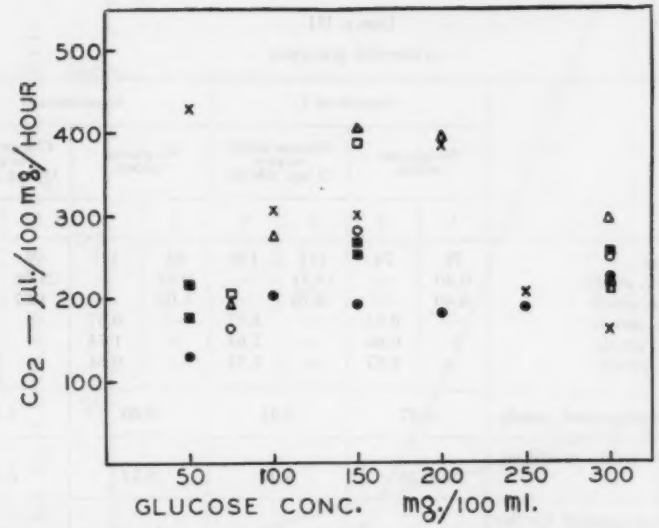

FIGURE 2. The rate of anaerobic glycolysis at different glucose concentrations. Media as described under Methods. Temp. 30.1° C. Each set of symbols represents the data obtained in any one experiment.

In order to make data of the type presented in Table III truly comparable the results can be expressed in terms of 100 mg. of tissue so as to rule out an inequality in the amount of tissue present in the two vessels of any one set. Such calculations, however, do not significantly alter the over-all pattern of the results. In a series of six experiments of the type given in Table III, where glucose was added and the results expressed in terms of 100 mg. of tissue, the following average values were obtained: 92% of the CO_2 release accounted for by lactic acid production; 85% of the glucose consumption accounted for by lactic acid production. In a series of five experiments where glucose was not added the corresponding average values were 80 and 74%, respectively.

In experiments where glucose was not added and the total changes are therefore small the results are quite variable as the data of Table III indicate, the two

experiments given in this table representing the extreme values of five such runs. From the average initial glucose in the vessels of these five experiments it may be calculated that 0.105 mg. of glucose was present for each 100 mg. of tissue, or roughly a value of 105 mg./100 ml. The average initial lactate concentration was

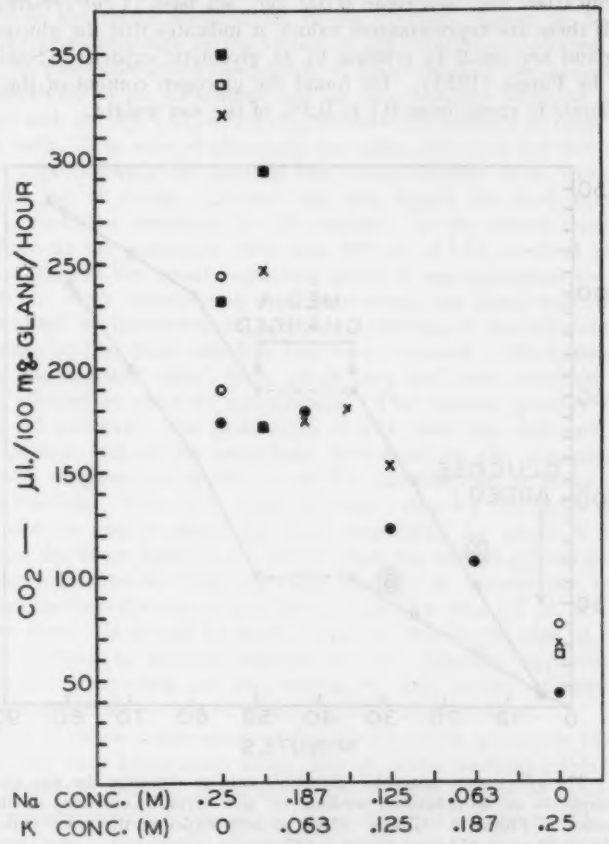

FIGURE 3. The effect of sodium and potassium on anaerobic glycolysis rates. The all-sodium medium was prepared as described under Methods. The all-potassium medium was prepared in the same manner except that the potassium salts were used instead of sodium. These two media were mixed in varying proportions to give media with varying ratios of the two cations. Glucose present in all cases to give an initial concentration of 150 mg. per 100 ml. Temp. 30.1° C.

0.060 mg. per 100 mg. of tissue. If it is assumed that the initial lactic acid was produced from glucose during the interval between removal of the gland from the fish and the time of analysis, then a total initial glucose value of 165 mg./100 ml. might be expected. Since this is much higher than the average value of

53 mg./100 ml. reported by Gray and Hall (1930) for the blood sugar of the scup, it suggests that glycogen breakdown is occurring. Dr. C. T. Teng kindly analyzed for us the glycogen content of the gas gland immediately after its removal from three species of fish. Expressed in terms of glucose per 100 mg. of tissue, he found: scup 0.097 mg., sea robin 0.102 mg., sea bass (*Centropristus striatus*) 0.070 mg. If these are representative values, it indicates that the glucose reserves of the gas gland are small in relation to its glycolytic capacity. Similar values are reported by Fänge (1953). He found the glycogen content of the gas gland in *Gadus callarias* to range from 0.1 to 0.3% of the wet weight.

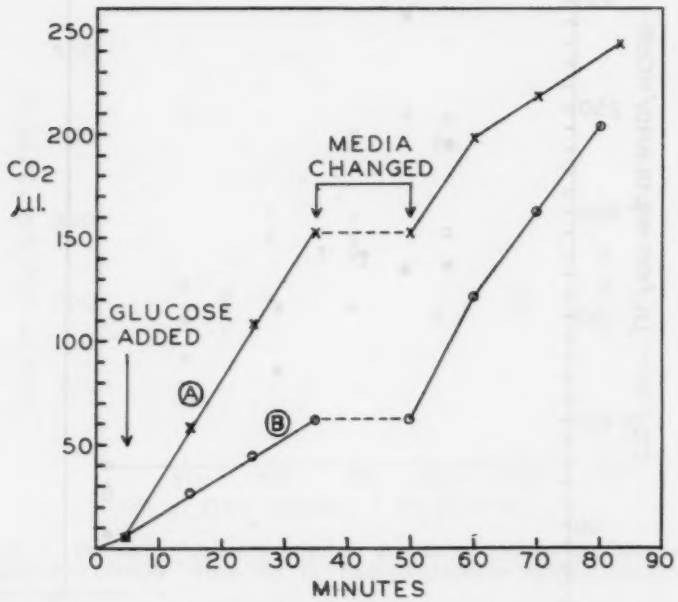

FIGURE 4. The effect upon anaerobic glycolysis rate of changing the gas gland from an all-sodium medium to an all-potassium medium or vice versa. Conditions as described for the data presented in Figure 3. Glucose added to both flasks at the point indicated by the arrow. Weight of Gland A 153 mg.; Gland B 147 mg.

A study of the effect of the concentration of glucose in the medium on the rate of glycolysis is portrayed in the data presented in Figure 2. There is a suggestion of a trend towards maximum glycolytic activity when the glucose concentration is 150 to 200 mg./100 ml., but the rate of glycolysis varies considerably from one gland to another at any one glucose concentration.

The cation composition of the medium appears to have a marked effect upon the rate of glycolysis. In Figure 3 are plotted the results of a series of experiments where the ratio of sodium to potassium in the medium was the only variable.

It can be seen that maximum rates are obtained in a medium containing all sodium ions while minimum rates occur when potassium is the single cation. Rates intermediate to these two extremes are obtained with media containing a mixture of Na and K. In these experiments there is on the average a four-fold increase in rate in changing from an all-potassium medium to an all-sodium one.

Changes in the cation composition of the medium appear to be rapidly reflected in the glycolysis rate as the results of the experiment plotted in Figure 4 illustrate. In this experiment two glands of approximately the same weight were placed respectively in an all-sodium and an all-potassium medium. The vessels were gassed with the 5% CO_2—95% N_2 mixture and allowed to come to temperature in the bath. The rate of glycolysis was then measured for five minutes and found to be approximately the same in both flasks, namely 48 μl. CO_2 evolved per hour per 100 mg. of tissue. Glucose was then tipped into both vessels and the rate of CO_2 evolution measured for 30 minutes. In the vessel containing gland A and sodium as the cation the rate was 190 μl. of CO_2 evolved per hour per 100 mg. of tissue; in the vessel containing gland B and potassium the comparable rate was 76 μl. Both vessels were then dismounted, the gland was removed from each and washed by immersion in a 0.25 *M* solution of the chloride salt of the cation opposite to that from which it had been removed. The glands were then returned to the opposite vessel from which they had been removed, the vessels gassed and allowed to come to temperature. The transfer process required approximately 15 minutes. The production of CO_2 was then followed for another 30-minute period. Gland B, previously immersed in the potassium medium, attained a rate of glycolysis of 194 μl. of CO_2 per 100 mg. of tissue per hour in the sodium medium. This is a value 2½ times that for the same gland in the potassium medium and is almost the same rate found for gland A in a sodium medium. On the other hand, it can be seen that the rate of glycolysis of gland A is slowed down by transfer from a sodium medium to a potassium medium. In the potassium medium the rate of glycolysis of gland A was 117 μl. of CO_2 per mg. of tissue per hour. It should be noted, however, that in the case of gland A the effect of its transfer to another medium becomes definitely apparent only after the first ten-minute reading and that during the last twenty minutes its rate of glycolysis is comparable to that of gland B in the same medium.

This effect of cation composition of the medium upon glycolysis rate prompted us to investigate what effect some other changes in the medium might have. The addition to a sodium medium of Ca and Mg salts, either alone or together, to give a 0.01 *M* final concentration of each of these salts had no appreciable effect upon the glycolysis rate. Inorganic phosphate added to a sodium medium to yield concentrations of 1, 4 or 10 mg. of P per 100 ml. also was without effect. Isotonic sea water (diluted 1 : 1) made 0.025 *M* in $NaHCO_3$ was as good a medium as an all-sodium one.

Variations in the molarity of an all-sodium medium within the range of 0.25 *M* to 0.0625 *M* had no appreciable effect upon the glycolysis rate.

The pH of the medium was varied by altering its $NaHCO_3$ content and holding the CO_2 tension constant. The results of a series of experiments are shown in Table IV. The rate of glycolysis increases as the pH rises. The pH values given are approximate and are calculated from the known values of the CO_2

tension and bicarbonate concentration since facilities were not available for the measurement of pH of solutions in equilibrium with a fixed gas phase.

A comparison of the rates of glycolysis with the four hexoses, glucose, mannose, fructose and galactose, was made. At concentrations of 150 mg./100 ml. the relative rates in the order named for these four sugars were 1.0, 0.86, 0.53 and 0.25. At 75 mg./100 ml. the respective relative rates were 1.0, 0.88, 0.30 and 0.14. These experiments were run in the standard sodium medium with one half of the same gland run in glucose and the other half run with the sugar under comparison. The relative rates reported by Christensen *et al.* (1949) for the action of the hexokinase of rat red blood cells upon glucose, mannose and fructose were 1.0, 0.77 and 0.36. Galactose was unreactive in their experiments. The purity of the sugars used in the experiments reported here is unknown to us.

TABLE IV

The effect of pH upon anaerobic glycolysis rate

$NaHCO_3$ content of medium	Calculated pH	$\mu l.$ CO_2/100 mg./hr.
12.25 mM	7.0	128*
		175
		138
		122
25.0 mM	7.3	273
		288
		327
		251†
50.0 mM	7.6	506
		315*
		397†
		303

*, † One half of the same gland was used in these experiments. Total molarity of medium in all cases made 0.25 *M* by appropriate addition of NaCl. Gas phase 5% CO_2–95% N_2, Temp. 30.1° C.

They were all commercial samples (Eastman Kodak Co.) and were used without any purification.

The glycolysis experiments reported so far have all been performed under anaerobic conditions (5% CO_2–95% N_2) in order to simplify the manometric calculations. Since the gland *in vivo*, especially in deep water fishes, must be in contact with oxygen at relatively high pressures, it seemed important to observe what effect the presence of oxygen might have upon the glycolysis rate. The results of experiments carried out under aerobic conditions (5% CO_2–95% O_2) are shown in Table V. These experiments were carried out in the same manner as those described for the anaerobic experiments reported in Table III. Here, however, the gas exchange values are of course complicated by the fact that oxygen consumption is proceeding at the same time that CO_2 evolution is occurring, both from metabolic oxidations and its release from bicarbonate in the medium by acid production. However, if the RQ of the respiration process is close to 1,

then respiratory CO_2 production will cancel out oxygen consumption manometrically and in the presence of glucose, at any rate, the manometric changes will primarily reflect the glycolysis process. In any case, the total oxygen consumption of the gland (*cf.* Table I) amounts on the average to 1.3 μmols per 100 mg. of gland per hour. This is approximately 20% of the amount of CO_2 produced in the experiments of Table V where glucose is present. The results reported in Table V clearly indicate that glycolysis proceeds readily under conditions where the dissolved oxygen is about equal to that found in water in equilibrium with air under five atmospheres pressure. Indeed the average lactate production, as

TABLE V

Aerobic glycolysis

	Experiment I				Experiment II			
	No glucose added		Glucose added to give 75 mg./100 ml.		Glucose added to give 75 mg./100 ml.			
Vessel	1	2	3	4	1	2	3	4
Gland wt., mg.	84	102	124*	133*	95	102	60	87
Initial glucose, μmols	0.00	—	11.34	—	11.61	—	11.83	—
Initial lactate, μmols	0.65	—	0.75	—	0.89	—	0.60	—
Final glucose, μmols	—	0.00	—	7.83	—	6.73	—	8.17
Final lactate, μmols	—	0.68	—	6.46	—	8.83	—	5.83
Gas consumed (−) or evolved (+), μmols	—	−0.23	—	+6.03	—	+8.58	—	+5.75
Total glucose consumed, μmols	—		3.51		4.88		3.66	
Total lactic acid produced, μmols	—		5.71		7.94		5.23	
CO_2 release accounted for by lactic acid production	—		95%		93%		91%	
Glucose consumption accounted for by lactic acid production	—		81%		81%		71%	

* One gland was cut approximately in half and used for these two vessels. Gas phase 5% CO_2–95% O_2. Temp. 30.1° C.

measured chemically, for four experiments run under aerobic conditions and with glucose added to give 75 mg./100 ml. was 6.04 μmols per 100 mg. of tissue per hour. Under anaerobic conditions at the same glucose concentration and temperature the average lactate production for three experiments was 5.81 μmols per 100 mg. per hour.

A few experiments were performed on homogenates of glands. Glycolysis does not proceed readily in homogenates. In an experiment where lactate production was measured chemically a value of 0.60 μmols per 100 mg. of tissue per hour was found. In a companion run where ATP was added a value of 0.67

μmols was found. These values are roughly 10% of those found for the intact gland. The addition of magnesium does not alter the glycolytic behavior of homogenates. Factors other than these have not been studied.

Discussion

It is of interest that Bohr in 1894 studied the "disengagement of gases in the air bladder of fishes." Ten years later, working on mammalian blood with Hasselbach and Krogh (1904), he described the effect which now bears his name, namely that the oxygen affinity of hemoglobin is markedly affected by the CO_2 tension, or acidity of the blood. One can find no mention that Bohr was aware that the two phenomena he had studied might be related. Some 15 years later Bohr's student Krogh (Krogh and Leitch, 1919), showed that fish blood showed an exceptionally marked Bohr effect. As far as we can ascertain, Hall (1924) was the first individual to investigate the possibility that a relationship might exist between the Bohr effect and oxygen secretion into the swim bladder. Hall placed freshly excised intact glands of the yellow perch in five ml. of distilled water and determined colorimetrically the pH of the water after an interval of usually 30 minutes and reported an average value of 7.05. He did the same for glands removed from fishes whose glands had been previously stimulated by withdrawal of gas and found on the average a pH value of 6.38. Hall states (p. 112) "acidity is evidently produced in the epithelial cells and acid dialyzes into the capillaries. . . . Just what the nature of the effective acid is can not be stated at present."

Since then the concept that the Bohr effect plays a role in the formation of gases in the swim bladder has received considerable attention. The results of Hall were repeated by Akita (1936). Black (1946) studied the chloride content of the gland in order to learn whether HCl might be the acid involved. The role of carbon dioxide and carbonic anhydrase in the process has been implicated by Leiner (1940) and investigated by many workers (*cf.* Fänge, 1953). However, to the best of our knowledge lactic acid has not heretofore been considered as the acidifying agent.

The ability of the gas gland to convert glucose into lactic acid is such that this tissue must be rated along with those like retina as outstanding in this capacity. Expressed in terms of $Q_G^{N_2}$, μl. CO_2 expelled from bicarbonate per dry weight of tissue per hour, the gas gland of scup, as studied here, has had a range of values from 12 to 23 at 30° C. For fish retina at 30° C. Nakashima (1928) reports an average value of 29. However, it is interesting to note that in the presence of 95% O_2 this same worker reports that the value drops to 1.0. This effect of oxygen upon glycolysis is one repeatedly observed for many tissues and has received the name of the Pasteur effect. (*cf.* Lipmann, 1942 and Burk, 1935). It is therefore of particular interest that in our studies the rate of glycolysis of the gas gland has been found to be unaffected by the presence of oxygen. Such an absence of the Pasteur effect in the gas gland would, of course, be essential if lactic acid production were to play a role in the secretion of oxygen, especially when it is considered that in deep sea fishes oxygen tensions in the swim bladder may reach values in the range of 100 atmospheres.

A schematic representation of events leading to the introduction of gas into the swim bladder has been made in Figure 5 based on the assumption that lactic acid production plays a central role in the process. The entering arterial blood is portrayed as carrying in oxyhemoglobin matched against some cation (*e.g.* potassium), glucose and $NaHCO_3$. On reaching the gland glucose diffuses into it and is converted into lactic acid. The latter acidifies the gland and in turn the blood. This acidification of the blood causes a dissociation of the oxyhemoglobin with the release of O_2 and also converts some $NaHCO_3$ into CO_2. The tensions of both these gases in the blood are thus increased and they tend to diffuse across the glandular structures and into the swim bladder. The venous blood is also, however, rich in these dissolved gases and a large part of the O_2 and CO_2 would

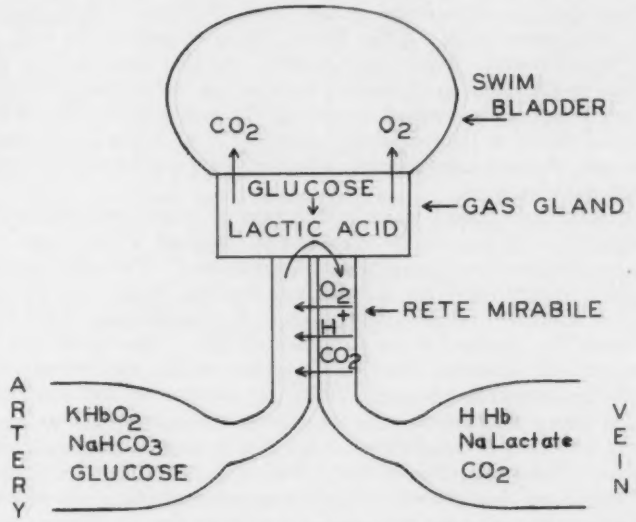

FIGURE 5. A schematic representation of the metabolism of the gas gland in relation to gas production and the role of the *rete mirabile*.

probably be carried away in solution in the blood if it were not for the *rete mirabile*. This amazing network of closely interwoven arterial and venous capillaries is portrayed to function as an exchange device along the lines suggested by earlier workers (*cf.* Jacobs, 1930). Here the outgoing venous blood equilibrates its diffusible components against the incoming blood. Thus O_2 and CO_2 tend to diffuse into the arterial blood and to presaturate the entering blood with these physically dissolved gases. The liberation of the bound O_2 and CO_2 by acidification as this presaturated blood reaches the gland thus further raises the pressure of these gases in the gland. As this cycle continues, higher and higher pressures will be built up which will facilitate the escape of gas into the swim bladder. It seems likely that there also will be a tendency for the H^+ concentration to equilibrate. There is thus also portrayed in Figure 5 the passage of H^+ ions from venous

to arterial blood in the *rete mirabile* which would cause a pre-acidification of the blood reaching the gland. Two other components whose concentration will differ in the venous and arterial blood are lactate and glucose. The latter is perhaps the more important since its equilibration would tend to lower the available supply of glucose to the gland. However, since the passage of glucose across the capillary wall of the rete may not be a simple diffusion process, no further consideration of this factor will be made at this time.

It is perhaps important to point out that such an acidification mechanism could account for the accumulation of both O_2 and CO_2 in the swim bladder. The CO_2 content of the swim bladder appears to be highest during periods just after the gland has been very active (Jacobs, 1930). We are not prepared at this time, however, to state categorically that such a mechanism alone is able to account for the very high gas pressures found in the swim bladders of fish taken at great depths (*cf.* Scholander *et al.*, 1951, 1953). It is of interest to note, however, that Jones and Marshall (1953) have pointed out that both the gas gland and the *rete mirabile* are much more highly developed in bathypelagic teleosts than in shallow water forms. The marked increase in the length of the rete of the deeper water forms as shown in their drawings is indeed most striking and suggests that the equilibration of venous and arterial blood plays an important role in the attainment of high gas pressures.

In the case of those fishes which also appear to concentrate nitrogen to some extent in their swim bladder (*cf.* Scholander and van Dam, 1953), some mechansim in addition to that presented would seem to be required. One could postulate that the hemoglobin of such fishes has the ability to combine slightly with N_2 as well as O_2 and that acidification releases both gases in a constant ratio depending on the species as found by Scholander and van Dam (1953). This would not, however, explain the unusual situation found in *Coregonus* where the nitrogen content of the swim bladder gases reaches nearly 100% (*cf.* Scholander and van Dam, 1953).

Assuming that a mechanism of the type proposed is in operation, a calculation is possible of the order of magnitude for the rate of oxygen production by the gas gland *in vivo*. The average blood sugar of 20 scup is reported by Gray and Hall (1930) to be 53 mg. per 100 ml. The average rate of glycolysis *in vitro* of the gas gland as determined here at a glucose concentration of 75 mg. per 100 ml. may be taken as 4.0 μmols or 0.72 mg. of glucose per hour per 100 mg. of tissue (*cf.* Table V). Thus, if the gas gland utilizes glucose at the same rate *in vivo*, it would require that at the minimum $0.72/53.0 \times 100$ ml., or 1.3 to 1.4 ml. of blood be supplied to 100 mg. of gland per hour. This assumes that all the glucose in the blood will be utilized, which seems doubtful. Let us assume, therefore, that the blood flow is twice this minimum value or 2.8 ml. per hour per 100 mg. of gland. The mammalian kidney has a blood flow of the order of 9.0 ml. per 100 mg. of tissue per hour (Best and Taylor, 1945). If we take the value given by Root (1931) for the O_2 content of scup blood as 7.3 vol.%, it is possible to calculate that 0.2 ml. of O_2 per hour is supplied to each 100 mg. of gland when it has a blood flow of 2.8 ml. per hour. The oxygen consumption of 100 mg. of gland *in vitro* is of the order of 0.03 ml. per hour (*cf.* Table I). There would thus remain 0.17 ml. of O_2 per hour to supply the swim bladder.

The time required to fill a swim bladder with oxygen on this basis can also

be calculated. Expressed as the average of eight determinations, we have found in the scup that the bladder volume is 4.6% of the body weight (*cf.* Jones and Marshall, 1953) and there are 9.1 ml. of bladder volume per 100 mg. of gas gland tissue. Thus, with an oxygen supply of 0.17 ml. per hour per 100 mg. of gland it would require 9.1/0.17 or about 54 hours for the scup to fill its swim bladder with 100% oxygen at one atmosphere of pressure.

These calculations are based on metabolism studies at 30° C. and are subject to criticism on this point as well as on many others. They do, however, furnish some clues as to the rate that might be expected if such a mechanism is in operation. A comparison with some *in vivo* data given in the literature is possible. Jacobs (1930) reports data on *Perca fluviatilis* which indicates that 2–4 days are required for this species to completely refill its swim bladder after it has been emptied by puncture. The gas in the swim bladder at this time was approximately 50% oxygen. Bohr (1894), working with *Gadus callarias*, found that replenishment after puncture was complete in 24–48 hours with the O_2 reaching, in some cases, 80% of the total re-formed gas. The value of 54 hours calculated above to completely fill the swim bladder in the scup with 100% oxygen is thus not very far out of line with these *in vivo* observations.

A similar set of calculations may be made for the amount of lactic acid poured into the blood by the gland, using the same premises as employed for the oxygen calculations. The result is that about 3 μmols of acid are added to each ml. of blood that passes through the gland. Scup blood contains about 4 μmols of bicarbonate per ml. (Root, 1931). The magnitudes of the other buffering components of scup blood are not known to us so a calculation of the expected pH shift in the blood is not possible at this time.[3]

Summary

1. Metabolic studies have been carried out on the gas gland of the swim bladder of the scup, *Stenotomus chrysops*, at 30° C.

2. The intact gland has a $Q_{O_2} = 2.0$ which is not appreciably altered by the addition of glucose. Homogenates of the gland have a much lower rate of oxygen consumption.

3. The intact gland converts glucose to lactic acid at a rapid rate. This conversion has been followed by measurement of glucose disappearance, lactic acid appearance and the release of CO_2 from a bicarbonate medium. Values of $Q_{N_2}^{CO_2}$

[3] Since submitting this manuscript for publication there have appeared two interesting articles bearing on the subject discussed here. In one, Scholander and van Dam (1954) present data which indicate that oxygen remains combined with some fish hemoglobins at high oxygen pressures even when the blood pH is lowered by the addition of lactic acid to a value of 5.7, or CO_2 tensions are increased. The effect of a combination of low pH and high CO_2 tension, such as might be encountered *in vivo*, is not reported. They interpret their results as throwing serious doubt on the participation of the Bohr effect in the accumulation of oxygen in the swim bladder. In the other, Scholander (1954) discusses in detail the possible function of the *rete mirabile* and by means of a mathematical analysis shows that very great pressures could be built up in the swim bladder if the rete acts as an exchange device. By the same token it would seem possible that a very marked acidification of the blood reaching the gland could also be achieved if both CO_2 and hydrogen ions were exchanged between the venous and arterial blood. It is obvious that data are sorely needed on the oxygen content of blood entering and leaving an active gland.

ranging from 12–23 have been found. Lactic acid appearance accounts for 70–90% of the glucose that disappears. The gland does not show a Pasteur effect since similar values are obtained under either aerobic or anaerobic conditions.

4. The rate of anaerobic glycolysis is greatest at an alkaline pH and is markedly influenced by the cation composition of the medium. Activities in an all-sodium medium are 3–4 times those in an all-potassium medium.

5. A schematic diagram of the possible role of lactic acid production in the events leading to the accumulation of gas in the swim bladder is presented and calculations on the rates of oxygen production to be expected under such circumstances are made.

LITERATURE CITED

AKITA, Y. K., 1936. Studies on the physiology of the swim bladder. *J. Fac. Sci. Tokyo Univ. (Ser. 4)*, **4**: 111–135.

BARKER, S. B., AND WILLIAM H. SUMMERSON, 1941. The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.*, **138**: 535–554.

BEST, C. H., AND N. B. TAYLOR, 1945. Physiological basis of medical practice, 4th Ed., p. 149. The Williams and Wilkins Company, Baltimore.

BLACK, V. S., 1946. Chloride, carbonic anhydrase and catalase in the tissues of the perch *Perca flavescens* (Mitchill). *Rev. Canadienne Biol.*, **5**: 311–318.

BOHR, C., 1894. The influence of section of the vagus nerve on the disengagement of gases in the air bladder of fishes. *J. Physiol.*, **15**: 494–500.

BOHR, C., K. A. HASSELBACH AND A. KROGH, 1904. Ueber einen biologischen Beziehung wichtiger Einfluss den die Kohlensäurespannung des Blutes auf dessen Säuerstoffbindung übt. *Skand. Arch. Physiol.*, **16**: 402–412.

BURK, D., 1935. A colloquial consideration of the Pasteur and neo-Pasteur effect. *In: Symposia on Quantitative Biology*, **7**: 420–459.

CHRISTENSEN, WILLIAM R., CALVIN H. PLYMPTON AND ERIC G. BALL, 1949. The hexokinase of the rat erythrocyte and the influence of hormonal and other factors on its activity. *J. Biol. Chem.*, **180**: 791–802.

FÄNGE, RAGNAR, 1953. The mechanisms of gas transport in the euphysochist swimbladder. Acta Physiologica Scandinavica, **30**: Supplementum 110.

GRAY, I. E., AND F. G. HALL, 1930. Blood sugar and activity in fishes with notes on the action of insulin. *Biol. Bull.*, **58**: 217–223.

HALL, F. G., 1924. The functions of the swim bladder of fishes. *Biol. Bull.*, **47**: 79–126.

JACOBS, W., 1930. Untersuchungen zur Physiologie der Schwimmblase der Fische. *Zeitschr. f. vergl. Physiol.*, **11**: 565–629.

JONES, F. R. HARDEN, AND N. B. MARSHALL, 1953. The structure and functions of the teleostean swimbladder. *Biol. Rev.*, **28**: 16–76.

KROGH, A., AND I. LEITCH, 1919. The respiratory function of the blood of fishes. *J. Physiol.*, **52**: 288–300.

LEINER, M., 1940. Das Atmungsfērment Kohlensäureanhydrase im Tierkörper. *Naturw.*, **28**: 165–171.

LIPMANN, F., 1942. Pasteur effect. *In: A symposium on respiratory enzymes*, pp. 48–73. University of Wisconsin Press.

NAKASHIMA, M., 1928. Stoffwechsel der Fischnetzhaut bei verschiedenen Temperaturen. *Biochem. Zeitschr.*, **204**: 479–481.

POTTER, V. R., AND C. A. ELVEHJEM, 1936. A modified method for the study of tissue oxidations. *J. Biol. Chem.*, **114**: 495–504.

ROOT, R. W., 1931. The respiratory function of the blood of marine fishes. *Biol. Bull.*, **61**: 427–456.

SCHOLANDER, P. F., AND L. VAN DAM, 1953. Composition of the swimbladder gas in deep sea fishes. *Biol. Bull.*, **104**: 75–86.

SCHOLANDER, P. F., C. L. CLAFF, C. T. TENG AND V. WALTERS, 1951. Nitrogen tension in the swimbladder of marine fishes in relation to the depth. *Biol. Bull.*, **101**: 178–193.

SCHOLANDER, P. F., AND L. VAN DAM, 1954. Secretion of gases against high pressures in the swimbladder of deep sea fishes. I. Oxygen dissociation in blood. *Biol. Bull.*, **107**: 247–259.

SCHOLANDER, P. F., 1954. Secretion of gases against high pressures in the swimbladder of deep sea fishes. II. The rete mirabile. *Biol. Bull.*, **107**: 260–277.

SOMOGYI, M., 1945. A new reagent for the determination of sugars. *J. Biol. Chem.*, **160**: 61–68.

STRITTMATTER, C. F., E. G. BALL AND O. COOPER, 1952. Glycolytic activity in the swim bladder gland. *Biol. Bull.*, **103**: 317.

A NOTE CONCERNING THE DISTRIBUTION OF POLYSACCHARIDES IN THE EARLY DEVELOPMENT OF THE HYDROMEDUSAN PHIALIDIUM GREGARIUM [1, 2]

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Since many organisms are actually supported by polysaccharides, that is, they form the framework for the architecture of the plant or animal, it was thought that it would be most interesting to follow the distribution of polysaccharides during the course of development, from egg to polyp, in the hydromedusan *Phialidium*. Fortunately, there is a simple and dependable histochemical technique to demonstrate these substances, namely, the periodic acid-Schiff method and, as will be shown, the non-starch polysaccharides are present in large amounts throughout the development and no special region of secretion of the external chitinous perisarc can be seen. On the other hand, there is a most striking and curious pattern associated with the attachment process of the planula larva.

Methods

The jellyfish were collected in the evening at a night light off the dock of the Laboratory of the University of Washington, at Friday Harbor, in Puget Sound. They were immediately placed in fresh sea water in finger bowls (100 mm. diameter) on the water table and kept at approximately 13° C. Six to a dozen jellyfish were placed in each bowl and these were removed the next day so that the eggs which had been shed could develop. For the attachment stages, two per cent agar in sea water was prepared to cover the bottom of clean finger bowls. Early planulae were transferred to these dishes, and when they attached to the agar, they could be easily removed without being damaged and transferred to the fixative.

The embryos were fixed in Bouin's fluid at 2° C., dehydrated in a series of graded alcohols, and embedded in paraffin. Polysaccharides were demonstrated by the periodic acid-Schiff technique (Gomori, 1952). The best results were obtained when a three-hour period of salivary digestion, which removes all the glycogen, preceded the staining. Control slides were run without the oxidation which showed an evenly distributed, very faint pink tinge.

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and J. T. Bonner, NR 164-274.

[2] It is possible that some of these observations were made on individuals of the closely related species, *P. hemisphericum*, for it is likely that the two species are both present in Puget Sound. Specific characters include the numbers of tentacles and marginal vesicles, and since these structures vary within rather wide limits, certain identification rests upon the examination of large numbers of individuals and the rearing of the hydroid stages.

I am indebted to Dr. D. L. Ray of the University of Washington for this information and for her kindness in reading the manuscript.

Results

As can be seen from Figure 1, in the egg there is a fairly even distribution of non-starch polysaccharide located primarily in large granules and this same pattern persists throughout cleavage as well as in the blastula stage. Even in the beginning of gastrulation, as the future endoderm cells wander into the blastocoel at one pole, there is no major difference among the cells in their staining intensity.

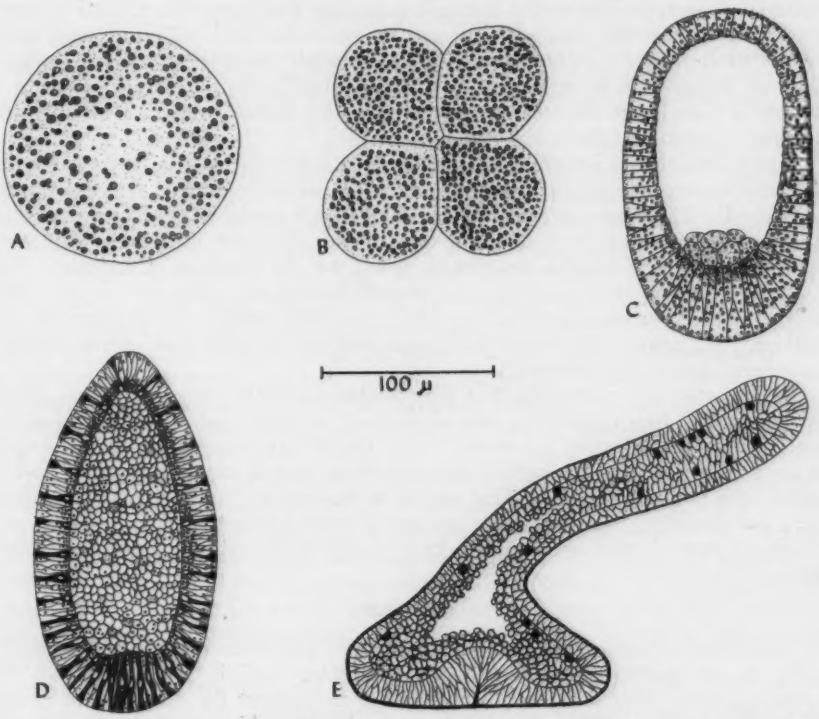

Figure 1. Drawings showing the distribution of non-starch polysaccharide in the development of *Phialidium*. The intensity of the black reflects the concentration of the polysaccharide. A, egg; B, 4-cell stage; C, 23 hours after fertilization showing the beginning of gastrulation by polar ingression (note that in this drawing and the next, the anterior end which attaches to the substratum is pointing downward); D, a late planula larva 60 hours after fertilization; E, a young attached larva just prior to the appearance of annulations in the chitinous perisarc.

It is only well after the gastrulation is complete and the motile planula shows contractile worm-like movements and ciliary activity, that there is a beautiful pattern of intensely stained goblet cells distributed within the entire ectodermal layer.

These cells are clearly more concentrated in the blunt anterior pole, in fact, in some larvae they appear so dense in that region that they almost lie side by side in a solid mass. The tapering, posterior end (which will later become the

hydranth end of the developing polyp) has few or none of these cells and there is a gradient of increasing goblet cell concentration as one passes anteriorly. In a surface view it is striking to see that these trumpet-shaped cells are spaced in an orderly fashion, each occupying the position farthest removed from all its neighbors. At this stage there are no especially intense staining regions in the endoderm.

Upon fixation the planula attaches its anterior end to the substratum and proceeds rapidly to flatten like a pancake. Almost immediately, hard chitinous material is secreted about this flattened mass and then some hours later a small nipple appears at the location that was the posterior end. This nipple, which is of a fixed diameter, continues to elongate (probably largely by mass cell migration or morphogenetic movement), showing annulations in the perisarc as it proceeds, and this is the stem or coenosarc of the future primary hydranth that eventually appears at the apical end.

Once fixation was accomplished and the first chitinous material surrounded the flattened embryo, there was no evidence of the intensely stained trumpet-shaped cells at all. They had apparently given off all their material in the fixation process, and we might assume from this that these gland cells have during the course of evolution been perfected in the planula solely for the function of fixation. The fact that planulae possess gland cells is well known and described in the early literature (see, for example, Wulfert, 1902) but their orderly distribution over the surface, their activity in relation to fixation and the production of polysaccharides was never specifically examined.

It is particularly surprising that the rounded tip of the young coenosarc which rises upward, does not have a special zone of secretion, for it is there that the chitinous wall is primarily deposited. The cells of this rising nipple appear evenly stained and, therefore, are high in polysaccharide content but they have no special gland cells nor any clearly defined region of deposition. In the endoderm, lying near the mesoglea, there are round masses of more intensely stained material, but because of their internal location, it is hard to understand what function they might have.

Summary

The distribution of non-starch polysaccharides was studied from egg to attached polyp in the hydromedusan *Phialidium gregarium* and it was found that the polysaccharides were fairly evenly distributed throughout the development except in the late planula. There a number of gland cells richly supplied with polysaccharide appear in the ectoderm and show a gradient—sparse in the posterior end and dense in the anterior attachment region. These cells are apparently concerned with fixation, for after the larva has attached and secretes a hard chitinous covering, these ectodermal concentrations of polysaccharide are no longer visible.

Literature Cited

Gomori, G., 1952. Microscopic histochemistry. University of Chicago Press, Chicago, Illinois.

Wulfert, J., 1902. Die Embryonalentwicklung von *Gonothyraea loveni* Allm. *Zeitschr. f. Wiss. Zool.*, **71**: 296–327.

THE EFFECT OF RADIATION FROM SMALL AMOUNTS OF P^{32}, S^{35} AND K^{42} ON THE DEVELOPMENT OF ARBACIA EGGS [1]

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In the recent extensive use of isotopes in biological research, little concern has been manifested for the effects of trace amounts of radioactive isotopes on cellular processes. Radioactive tracer studies in whole animals are usually not controlled at the level they seek to elucidate and few studies involving single cells have been designed to determine minimum levels of radioactivity which affect synthetic and developmental processes. Kamen (1947) states (p. 125), "It must be emphasized that tracer radiations in themselves constitute an ever present hazard in research on metabolism because marked physiological effects invalidating conclusions can arise if high tracer concentrations are used." Unfortunately, as he points out, little systematic information on the effects of low radiation dosage is available and what knowledge exists indicates marked variability in sensitivity in different forms. The dose range to produce various biological effects varies from 10 to 10^6 roentgens (Patt, 1953).

Thus, although our knowledge of radiation effects is large (see Henshaw, 1944; Lea, 1947; and Gray, 1952), little attention has been devoted to a study of the effects of ionizing radiations emitted from trace amounts of radioactive isotopes upon single cells. That small amounts of radiation may have significant effects on *Paramecium* has been demonstrated for x-radiation by Hance and Clark (1925), and more recently for β-radiation by Daniel and Park (1953). Packard (1916) reported acceleration of cleavage in *Arbacia* treated with short exposures to radium.

Mullins (1939), working with *Nitella*, found that concentrations of Na^{24} above one microcurie (μC) per ml. decreased the amount of ion penetration into these cells. Brooks (1943) determined the uptake of P^{32} and Na^{24} in *Arbacia* and *Asterias* eggs. His P^{32} concentrations ranged from 0.045 to 0.29 μC per ml. and because the amount of β-radiation emitted by the P^{32} was less than that observed by Mullins (1939) to affect *Nitella* permeability, he assumed that the radiation was not a factor in his measurements. In a later study (Brooks and Chambers, 1948) the P^{32} uptake in several species of Pacific coast sea urchins was investigated using concentrations of 0.001 to 0.3 μC per ml.

The present study was undertaken to determine the dosage levels of P^{32}, S^{35} and K^{42} upon eggs or sperm which would have an effect on the cleavage and subsequent development of *Arbacia* eggs. The results indicate that radiation from

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P^{32}, S^{35} and K^{42} at dose levels ranging from 0.25 to 50 μC per ml. (which includes the range used by others for permeability studies) has an accelerating or retarding effect on the first cleavage, depending upon the dose, and also retards subsequent development of the plutei.

Methods

Eggs and sperm of *Arbacia punctulata* were prepared in the manner described by Blum and Price (1950).

The isotopes used in this study were S^{35}, P^{32} and K^{42}.[2] Sea water solutions of each of these were prepared by evaporating carrier-free isotope solutions to dryness and dissolving the isotope residue in sea water. The pH of such solutions was the same as that of sea water. Stock solutions of S^{35} were prepared weekly in concentrations of 0.5, 5.0 and 50 μC per ml. P^{32} solutions were prepared in the same concentrations and used within two days. The K^{42} solutions were prepared in a concentration of one μC per ml. and used immediately.

The experiments were performed in the following manner: four 25-mm. stender dishes were placed in running sea water to a depth of one cm. to keep the contents at the temperature of the sea water. To dish one was added one ml. of filtered sea water; to dishes two, three and four were added one ml. of filtered sea water containing isotope concentrations of 0.5, 5.0 and 50 μC per ml., respectively.

When the object of the experiment was the irradiation of sperm, one or two drops of dilute sperm suspension were added to each of the dishes. Generally after two hours, one ml. of freshly washed eggs was then added, the time noted, and samples of the cleaving eggs taken at two-minute intervals for 14 minutes after cleavage began in the control. One-drop samples were preserved in 5 per cent formalin in sea water in shell vials for later counting. The per cent cleavage for a given time interval was determined by counting 100–200 eggs in each sample period. Egg irradiation experiments were performed in the same manner as the sperm experiments. One ml. of a dilute suspension of freshly washed eggs was added to each dish. Such additions diluted the isotope concentrations by one half so that eggs were exposed to 0.25, 2.5 and 25 μC of radioactivity per ml. Doubling the concentration of the eggs had no effect on the results. All experiments were carried out in duplicate. The temperatures of the control and experimental solutions varied from 22° to 24° C. but did not vary more than one half degree C. during any one experiment.

Irradiated sperm were always fertilized with non-irradiated eggs and irradiated eggs with non-irradiated sperm. No experiments were performed in which irradiated eggs were fertilized with irradiated sperm. After preserving samples of the first cleavage, the remaining zygotes in the dishes were permitted to develop. When control forms had reached the pluteus stage, usually after 48–72 hours, samples of all dishes were preserved in formalin sea water for comparison. It should be noted that after fertilization the developing larva remained in the same isotope environment as the irradiated gametes.

[2] The isotopes used in the study were obtained on allocation from the Isotopes Division, United States Atomic Energy Commission. The authors would like to acknowledge the helpful assistance of Mr. S. Berman in preparation of the isotope solutions.

Results

Preliminary experiments utilizing P^{32} had been previously carried out and reported upon (Green and Roth, 1950), but the data reported here were obtained during the summer of 1953.

Figure 1 is a plot of the per cent first cleavage against time of *Arbacia* eggs fertilized with sperm which had been irradiated for two hours prior to fertilization by three dose levels of S^{35}. The graph shows that the time of cleavage is delayed

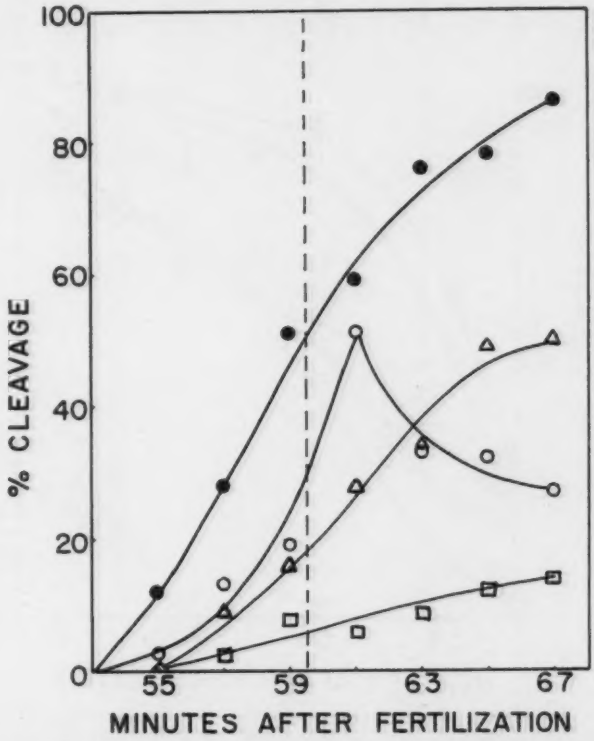

Figure 1. Plot of per cent first cleavage against time when sperm were irradiated for two hours prior to fertilization of non-irradiated eggs. ● = non-irradiated; ○ = 0.5 μC per ml. S^{35}; △ = 5.0 μC per ml. S^{35}; □ = 50 μC per ml. S^{35}.

by prior irradiation of the sperm, roughly in proportion to the dosage of radioactivity. The decrease in per cent cleavage in the case of the 0.5-μC dose after 61 minutes does not represent an error in counting but is probably attributed to experimental variation.

Figure 2 is a plot similar to Figure 1 and shows the results of irradiation of eggs for two hours with three dose levels of S^{35} prior to fertilizing with non-

irradiated sperm. Although the dose levels shown in Figure 2 are different from those of Figure 1, there is some overlapping and one can readily see that egg irradiation prior to fertilization has a less marked retarding effect on subsequent cleavage than does the irradiation of sperm. Figure 2 shows that the lowest dose level had an accelerating effect on cleavage which may not be too significant. The vertical dotted line appearing in both Figures 1 and 2 indicates the per cent

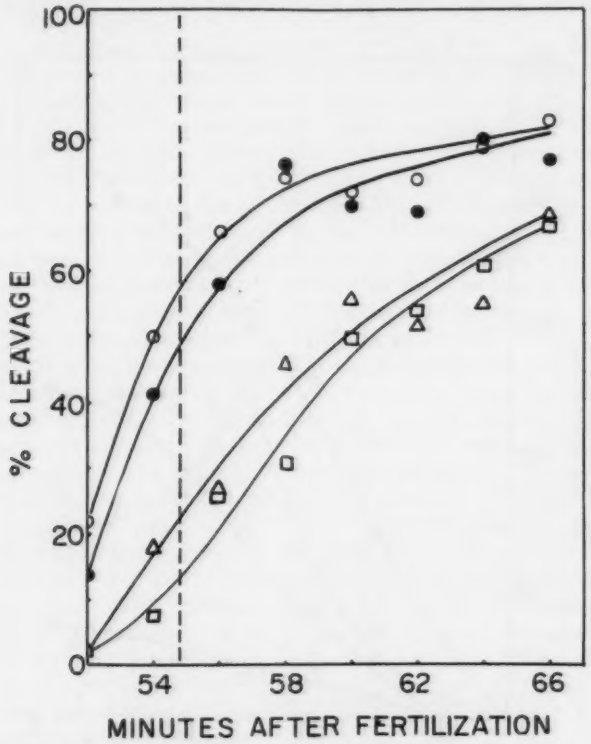

FIGURE 2. Plot of per cent first cleavage against time when eggs were irradiated for two hours prior to fertilization with non-irradiated sperm. ● = non-irradiated; ○ = 0.25 μC per ml. S^{35}; △ = 2.5 μC per ml. S^{35}; □ = 25.0 μC per ml. S^{35}.

cleavage attained by the experimental cells when the control cells had reached 50 per cent cleavage.

The data presented in Table I were obtained from graphing the results of a number of experiments in the manner of Figures 1 and 2. The amount of retardation or acceleration of cleavage by the irradiation of unfertilized gametes is presented as the per cent cleavage of the experimentally treated eggs at that time when 50 per cent of the control eggs had cleaved. This method of expressing the results was chosen because most of the egg suspensions treated with the higher

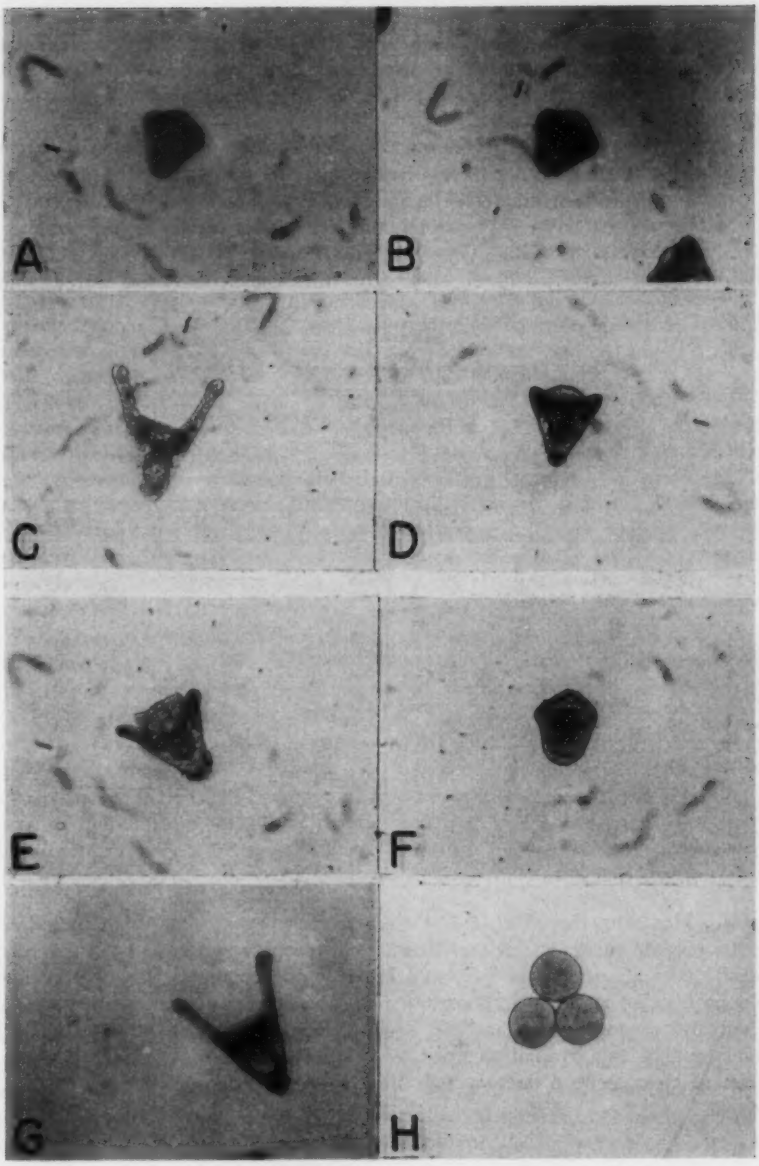

FIGURE 3. For explanation see text under "Results."

radiation doses did not attain 50 per cent cleavage during the period of observation. In general, duplicate experiments with eggs and sperm from different female and male *Arbacia* are given in the table. And, although the results from such experiments show variation, the direction and order of magnitude of the effects were frequently duplicated.

Figure 3 shows a series of photographs of developing larvae of *Arbacia* in which the effects of sperm and egg irradiation are compared. Photographs A, C, E and G are of non-irradiated cells and should be matched with photographs B, D, F and H, respectively, of corresponding irradiated cells. Egg irradiation is shown by photograph B (0.25 μC per ml. of S^{35}) and F (0.25 μC per ml. of P^{32}). In neither case are the plutei strikingly different from their corresponding

TABLE I

Effect of ionizing radiations on Arbacia egg cleavage

Date	Exp. No.	Isotope	Gamete irrad.	Prefert. expos. hrs.	Per cent of experimental eggs cleaved when 50% of controls have cleaved						
					Exposure dosage—microcuries/ml.						
					0.25	0.5	1.0	2.5	5.0	25.0	50.0
8/29/53	14A	P^{32}	Egg	2	60			43		27	
8/29/53	14B	P^{32}	Egg	2	55			50		15	
8/28/53	13	P^{32}	Sperm	2		14			0		0
7/22/53	5A	S^{35}	Egg	0	61			40		74	
7/13/53	2A	S^{35}	Egg	1	55			47		40	
7/13/53	2B	S^{35}	Egg	1	40			40		25	
7/17/53	4A	S^{35}	Egg	2	60			21		15	
7/17/53	4B	S^{35}	Egg	2	67			60		63	
7/24/53	6A	S^{35}	Sperm	2		25			15		5
7/24/53	6B	S^{35}	Sperm	2		32			13		6
8/22/53	9B	K^{42}	Egg	2		30					
8/21/53	10A	K^{42}	Sperm	2			10				
8/21/53	10B	K^{42}	Sperm	2			14				

controls. However, the effect of P^{32} was somewhat greater, as might be expected from the greater energy of its β-radiation. Sperm irradiation is shown by photographs D (0.5 μC per ml. of S^{35}) and H (0.5 μC per ml. of P^{32}). Both photographs indicate an appreciable retardation in growth as compared with corresponding controls (photographs C and G). These pictures support the data in Table I in showing that egg irradiation prior to fertilization has a less marked effect on subsequent cleavage and development than sperm irradiation.

DISCUSSION

It is rather difficult to calculate the exact exposure in terms of roentgens or equivalent roentgens (e.r.) of an individual egg or spermatozoan. The total

radiation dose in the solution during two hours, when radiation equilibrium obtains, may be calculated by the methods of Marinelli, Quimby and Hine (1948). For concentrations of 0.5, 5.0 and 50 μC of P^{32} per ml. this would be 1.74, 17.4 and 174 e.r., respectively, and for S^{35}, 0.139, 1.39 and 13.9 e.r., respectively. However, because of the small volume and thickness of solution used, the condition of "radiation equilibrium" was not fulfilled (especially in the case of P^{32}) and therefore the doses actually received by the eggs or the sperm were considerably less than those given above. This is particularly true in the case of the eggs, which lay at the bottom of the dishes and consequently were irradiated essentially from one side. In the case of the developing larvae in the presence of the higher concentrations of isotope for from 48 to 72 hours, the radiation dose might be appreciable, however. Daniel and Park (1953) found that solutions of S^{35} delivering from 0.77 to 3.13 e.r. per hour under radiation equilibrium conditions, stimulated *Paramecium* division. Their dose levels are well within the range of activities observed by us to be stimulatory to the *Arbacia* egg.

The date reported by Table I clearly show that cleavage delay results on irradiation of sperm with low levels of β-radiation. This finding is supported by the work of Mitueo *et al.* (1939) on eggs and sperm of *Pseudocentrotus depressus* exposed to β-rays from radium emanation, and was found to be true for *Arbacia* gametes irradiated with x-rays (Henshaw and Francis, 1936). Both Henshaw and Mituo irradiated testicular mashes and their results are subject to possible effects of so-called "necrohormones" (Heilbrunn and Young, 1935). Our studies were performed, however, on quite dilute sperm suspensions so that the indirect effects observed by Heilbrunn are not applicable.

The effects of low dose levels of β-radiation upon *Arbacia* zygotes and developing embryos cannot be determined by the results of Table I or Figure 3. The retardation of cleavage from sperm irradiation is continued into the pluteus stage but as all zygotes (egg-irradiated ones also) remained in the radiation environment of the gametes, the effects of gamete irradiation and zygote irradiation cannot be separated. One possible exception to this is the experiment performed in which *Arbacia* plutei were obtained after K^{42} irradiation of gametes. In this case because of the short life of this isotope, radiation levels could be expected to fall well below injury levels before plutei were formed. Egg irradiation had no effect on plutei formation in this experiment, while sperm irradiation resulted in the development of no plutei, although some initial cleavage occurred.

The knowledge that trace dose levels of these isotopes may cause biologically abnormal effects in *Arbacia* cannot be readily transferred to other types of cells. But this knowledge does carry the implication that rapidly dividing cells in the presence of radioisotopes, or, more important, cells which accumulate isotopes, may be modified in a subtle manner by the radiation emitted by these isotopes, even though the particular process studied by the investigator does not appear to be altered. A greater awareness of potential cell damage from radioactive tracers should lead to further investigations and to more systematic knowledge of limitations of these tools in biological research.

The authors would like to acknowledge the helpful assistance of Miss Mary Hodge in performing the egg counts.

SUMMARY

Arbacia gametes (eggs or sperm), exposed to radiation from S^{35}, P^{32} and K^{42} at dose levels ranging from 0.25 to 5.0 μC per ml. and subsequently fertilized with non-irradiated gametes, formed zygotes whose first cleavage was accelerated or retarded, depending upon the dose, and whose further development was slowed at the higher dose levels. Some experimental exceptions to these findings are reported and discussed. The implications of the results for biological tracer methodology are stated.

LITERATURE CITED

BLUM, H. F., AND J. P. PRICE, 1950. Delay of cleavage of the *Arbacia* egg by ultraviolet radiation. *J. Gen. Physiol.*, **33**: 285–303.

BROOKS, S. C., 1943. Intake and loss of ions by living cells. I. Eggs and larvae of *Arbacia punctulata* and *Asterias forbesi* exposed to phosphate and sodium ions. *Biol. Bull.*, **84**: 213–225.

BROOKS, S. C., AND E. L. CHAMBERS, 1948. Penetration of radioactive phosphate into the eggs of *Strongylocentrotus purpuratus, S. franciscanus,* and *Urechis caupo. Biol. Bull.*, **95**: 262–263.

DANIEL, G. E., AND H. D. PARK, 1953. Reproduction in *Paramecium* as affected by small doses of X-ray and β-radiation. *Proc. Soc. Exp. Biol. Med.*, **83**: 662–665.

GRAY, L. H., 1952. Biological damage induced by different types of ionizing radiations. In: *Biological Hazards of Atomic Energy,* edited by A. Haddow. Oxford University Press.

GREEN, J. W., AND J. S. ROTH, 1950. The effect of P^{32} on the division time of *Arbacia* eggs. *Biol. Bull.*, **99**: 358.

HANCE, R. T., AND H. CLARK, 1925. The effect of X-rays on the division rate of *Paramecium. J. Exp. Med.*, **43**: 231–240.

HEILBRUNN, L. V., AND R. A. YOUNG, 1935. Indirect effects of radiation on sea urchin eggs. *Biol. Bull.*, **69**: 274–278.

HENSHAW, P. S., 1944. Roentgen and Gamma rays: Biologic effects. In: *Medical Physics,* Edited by Otto Glasser, vol. 1, pp. 1352–1360. Year Book Publishers, Chicago, Ill.

HENSHAW, P. S., AND D. S. FRANCIS, 1936. The effect of X-rays on cleavage in *Arbacia* eggs: Evidence of nuclear control of division rate. *Biol. Bull.*, **70**: 28–35.

KAMEN, M. D., 1947. Radioactive tracers in biology. Academic Press, Inc., New York.

LEA, D. E., 1947. Actions of radiations on living cells. Cambridge University Press, Cambridge, England.

MARINELLI, L. D., E. H. QUIMBY AND G. H. HINE, 1948. Dosage determination with radioactive isotopes. II. Practical considerations in therapy and protection. *Amer. J. Roentgenology and Radium Ther.*, **59**: 260–280.

MITUO, M., H. YAMASHITA AND K. MORI, 1939. The action of ionizing rays on sea urchins. I. The effects of roentgen, gamma and beta rays upon the unfertilized eggs and sperm. *"Gann," The Jap. J. of Cancer Res.*, **33**: 1–12.

MULLINS, L. S., 1939. The effect of radiation from radioactive indicators on the penetration of ions into *Nitella. J. Cell. Comp. Physiol.*, **14**: 403–405.

PACKARD, C., 1916. The effect of radium radiations on the rate of cell division. *J. Exp. Zool.*, **21**: 199–212.

PATT, H. M., 1953. Protective mechanisms in ionizing radiation injury. *Physiol Rev.* **33**: 35–76.

SYNTHESIS OF DESOXYRIBONUCLEIC ACID IN LETHAL AMPHIBIAN HYBRIDS[1]

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It has been found that by cross-fertilization between certain combinations of related amphibian species lethal hybrids arise (Baltzer, 1934; Brachet, 1944; Moore, 1946). These lethal hybrids stop developing at a stage corresponding morphologically to early gastrula. Microscopical examination has shown that various abnormalities occur in the hybrid embryos, *e.g.* irregular mitoses and pycnosis (Schönmann, 1938; Brachet, 1944). When the controls have reached stage 13 (early neurula) all the cells, except those in the blastoporal region, have died (Schönmann, 1938; Moore, 1948).

The results of Hoff-Jørgensen and Zeuthen (1952; *cf.* also Løvtrup, 1954) have disclosed that no increase in total DNA occurs during the early development, up to a late blastula stage. This can only mean that the DNA used in the formation of new nuclei during the early stages of development must be present as a reserve in the mature oocyte. Hoff-Jørgensen and Zeuthen could demonstrate that the size of this store corresponds to the DNA content in about 2700 diploid nuclei in *Rana platyrrhinus*, and that it, to a major extent, is localized in the cytoplasm. It is a quite obvious suggestion that the developmental block observed in hybrids may reflect the lack of ability to synthesize substances necessary for continued development. On the basis of the observations mentioned above it would seem possible that DNA might be one of these substances. In that case one might expect the hybrid embryos to develop until the DNA reserves were exhausted. Although there is a discrepancy as to the morphological stage at which the DNA exhaustion and the developmental block occur, it was still considered worth while to test this possibility.

A necessary condition for doing this work was that the DNA analyses were carried out by the microbiological assay of DNA (desoxyribosides) worked out by Hoff-Jørgensen (1951), as the other available methods of DNA determination seem to be too unspecific or too insensitive to estimate DNA when present in such low concentrations as in early amphibian embryos (*cf.* the discussion by Løvtrup, 1954).

Material and Methods

Embryos

Normal *Rana pipiens* embryos, and *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrid embryos obtained by stripping *R. pipiens* eggs directly from the same female

[1] Supported by a grant from the Carlsberg Foundation.

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donors of control eggs into suspensions of *R. sylvatica* sperm, were reared in 10% Ringer's solution, without bicarbonate or phosphate, at a temperature of 15° C.

Preparation of dry samples

A single tube of dry material was prepared as follows. Jelly was removed with forceps from 25 embryos, which were then pipetted into a 10 × 75 mm. test tube. Excess medium was removed, and the tube nearly filled with acetone. The embryos were then crushed thoroughly with a ball-tipped glass rod. The tube was then centrifuged, the supernatant decanted, the precipitate re-suspended in acetone, re-centrifuged, decanted again, and left in a desiccator over calcium chloride for 24 hours. The open end of the tube was then drawn out and sealed off, and the tube stored until analyses for DNA could be made.

Hybrid and control embryos were prepared simultaneously. Each tube was labelled with the developmental stage number (Shumway, 1940) of the controls and with a designation of the time (in hours) elapsing since fertilization.

Determination of DNA [3]

The principle of the microbiological DNA assay is that a certain strain of lactic acid bacteria, *Thermobacterium acidophilus* R 26, is unable to grow in the absence of desoxyribosides. The extent of growth is almost proportional to the concentration of these substances in the culture medium. By measuring the growth obtained after adding an aliquot of the biological material to a culture medium free of desoxyribosides, it is possible to estimate the content of DNA. Before the microbiological assay is carried out the DNA in the samples is first hydrolyzed by the following procedure: To each tube of dry material is added 0.5 ml. of 0.5 N NaOH, and the tube placed in a boiling water bath for 15 minutes. After this is added 2.5 ml. buffer-activator solution (0.06 *M* maleic acid; 0.01 *M* $MgSO_4$), and the pH is checked and adjusted to 6.5–7.0 if necessary. Then 0.1 mg. crystalline desoxyribonuclease, contained in 0.1 ml. is added, and the tubes incubated 20 hours at 37° C. After this the sample is transferred to a volumetric flask, the volume made up to 5 ml., and DNA assayed as described by Hoff-Jørgensen (1951).

It may be argued that this method is not specific for DNA, as it determines the sum of DNA and free desoxyribosides. It should be noticed, however, that determinations on extracts not treated with desoxyribonuclease generally give negligible values (*cf.* the discussion following the paper by Hoff-Jørgensen, 1954). This observation thus leads to the not surprising conclusion that free desoxyribonucleosides are rarely found in the cells, even in embryos. It seems reasonable, therefore, to apply the name "DNA" to the substances determined by the present method, namely, those which liberate desoxyribonucleosides upon treatment with desoxyribonuclease. This does not imply anything with respect to the degree of polymerization of the substances, about which nothing is known. However, there is certainly no reason to believe that it is present in a high-polymeric form, ready for incorporation in the nuclei arising during segmentation. From a genetical point of view it would rather seem required that of the DNA incorporated during

[3] All the determinations of DNA were carried out in the laboratory of Dr. E. Hoff-Jørgensen. We gladly acknowledge our gratitude for this assistance.

the early development at least 50 per cent, *viz.* the DNA in the male chromosomes, be synthesized from rather low-molecular substances. The constancy of DNA during segmentation should not, therefore, be taken to demonstrate that no DNA synthesis occurs. Rather it indicates that DNA is synthesized at the expense of preformed desoxyribosides, and that no *total* synthesis of DNA occurs.

Results

The results are shown in Figure 1. The DNA content at the beginning of development is 0.063 ± 0.002 μg (standard deviation: ± 0.005 μg). This value is calculated as the average of five determinations (only three of these are shown

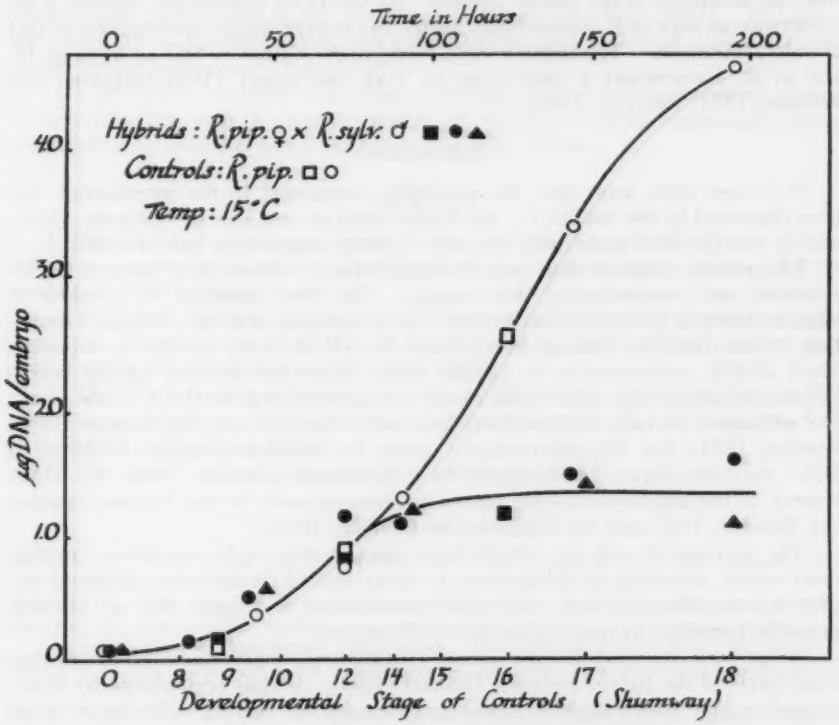

Figure 1. Desoxyribonucleic acid content of normal and hybrid embryos at various stages of development.

in the figure) on eggs from 2 to 4 hours after fertilization (2–8 cells). The content is the same in hybrids (0.065; 0.065) and in controls (0.068; 0.059; 0.058). According to Sze (1953) the DNA content of a diploid nucleus in *R. pipiens* is 10.4×10^{-6} μg. The reserve would thus correspond to about 6000 nuclei.

At the end of segmentation the content of DNA begins to rise in both hybrids and controls, the rate of synthesis increasing gradually for about 50 hours, *i.e.*, until the controls have reached stage 14 (Shumway). At this time the synthesis of DNA ceases in the hybrids, the final content calculated as the average of the last five values being 1.31 ± 0.08 μg DNA (standard deviation ± 0.17 μg). In the controls synthesis continues at constant rate (linear increase) until between stages 17 and 18, when a decrease in rate of synthesis is observed. There are rather few points on the control curve, but the shape of the curve is exactly the same as found in *R. platyrrhinus* (Hoff-Jørgensen and Zeuthen, 1952; Løvtrup, 1954). It should also be mentioned that the absolute amounts are approximately the same in the two species; thus the content in the oocyte of the European species is about 0.065 μg according to the former authors. As the DNA content per nucleus is almost twice as high in *R. platyrrhinus* nuclei, the reserve supply corresponds to that of only 2700 nuclei. The highest value found in *R. pipiens* is 4.62 μg at stage 18, and in *R. platyrrhinus* a value close to 5 μg was found (Hoff-Jørgensen and Zeuthen, 1952; Løvtrup, 1954).

Discussion

It is seen right away that the possibility mentioned in the introduction has been disproved by the results, *i.e.*, the hybrid embryos are able to synthesize DNA, and do so at normal speed until the early neurula stages have been reached.

The results obtained thus clearly demonstrate a dissociation between DNA synthesis and morphological development. The next question to consider is whether there is a dissociation between DNA synthesis and cell division (formation of new nuclei). During development the DNA of the hybrids is increased about 20-fold, corresponding to 120,000 cells. Were cell division lagging behind DNA synthesis to any appreciable extent, an accumulation of DNA would occur. No indication of such accumulation has been observed (*cf.* Schönmann, 1938; Brachet, 1952), but this unfortunately cannot be considered decisive evidence, as DNA may not always be detectable by cytochemical methods. Thus the DNA reserve in the amphibian oocyte cannot be demonstrated by the Feulgen reaction (*cf.* Brachet, 1952, and the discussion in Løvtrup, 1954).

The decrease in cell size which takes place during early amphibian development seems, according to Schönmann, to occur also in the hybrids. Without settling this question definitely, this observation seems to suggest that cell division proceeds normally, in spite of the abnormal mitoses.

It has been observed that the animal (blastoporal) region survives longer than other parts of the hybrid embryos (Baltzer, 1934). It has been shown by transplantation that both inductive power and capacity for further differentiation are not lost (Lüthi, 1938; Brachet, 1944; Moore, 1947, 1948). As the DNA synthesis proceeds normally in the hybrids until the early neurula stages, it would seem reasonable to suggest that this synthesis corresponds to cell divisions localized in the blastoporal (animal) region. The major part of the cells formed here will in the normal embryo invaginate and form the mesoderm. If this interpretation is correct, it seems possible from our results to divide the pre-larval DNA synthesis (cell division) into two phases, the first of which (mesodermal) is found in both hybrids and controls. The initiation of the second (linear) phase ap-

parently is conditioned by a normal gastrulation. It would seem that this second (ectodermal) phase to some extent is associated with the development of the neural tissues, epidermis, etc. It is interesting in this connection to note that a third phase of DNA synthesis, correlated at least partly with the development of the endoderm, has been found to begin during early larval development (*cf.* Løvtrup, 1954).

Much work has already been devoted to the study of the physiology and biochemistry of hybrid development. Thus the results of Steinert (1951) indicate that RNA also is synthesized in hybrid embryos, although to a lesser extent than in the controls. This finding is in agreement with the fact that very little RNA is synthesized before neurulation. In normal embryos a considerable increase in respiratory rate occurs during gastrulation. Contrary to this, only a very slight increase has been found in the hybrids (Barth, 1946; Chen, 1952). In agreement with this is the observation that carbohydrate is consumed at a very low rate in the hybrid embryos (Gregg, 1948).

It is difficult to correlate these various findings, but it seems warranted at least to conclude that only a minor fraction of the total energy consumed during gastrulation is used for DNA synthesis and cell division.

Summary

1. The DNA contents of normal *R. pipiens* embryos and *R. pipiens* ♀ × *R. sylvatica* ♂ embryos at various developmental stages have been estimated by the method of Hoff-Jørgensen (1951).

2. The DNA content of *R. pipiens* embryos remains constant from fertilization until near the end of segmentation, at which point it increases at a constant rate until shortly before muscular movements begin.

3. The DNA content of hybrid embryos (which do not gastrulate) is identical with that of *R. pipiens* embryos until the latter reach the neural fold stage, at which point it does not increase further.

4. The significance of these results is discussed briefly.

LITERATURE CITED

Baltzer, F., 1934. Ueber die Entwicklung der Bastardkombination *Triton palmatus* ♀ × *Salamandra maculosa* ♂. *Rev. Suisse Zool.*, **41**: 405–406.

Barth, L. G., 1946. Studies on the metabolism of development. *J. Exp. Zool.*, **103**: 463–486.

Brachet, J., 1944. Acides nucléiques et morphogénèse au cours de la parthenogénèse, la polyspermie et l'hybridation chez anoures. *Ann. Soc. Roy. Zool. Belg.*, **74**: 49–74.

Brachet, J., 1952. The role of the nucleus and the cytoplasm in synthesis and morphogenesis. *Symp. Soc. Exp. Biol.*, **6**: 173–200.

Chen, P. S., 1952. The rate of oxygen consumption in the lethal hybrid between *Triton* ♀ and *Salamandra* ♂. *Exp. Cell Res.*, **5**: 275–287.

Gregg, J. R., 1948. Carbohydrate metabolism of normal and of hybrid amphibian embryos. *J. Exp. Zool.*, **109**: 119–134.

Hoff-Jørgensen, E., 1951. A microbiological assay of desoxyribonucleosides and desoxyribonucleic acid. *Biochem. J.*, **50**: 400–403.

Hoff-Jørgensen, E., 1954. Desoxynucleic acid in some gametes and embryos. *In:* J. A. Kitching, Recent Developments in Cell Physiology, London, 1954, pp. 79–90.

Hoff-Jørgensen, E., and E. Zeuthen, 1952. Evidence of cytoplasmic desoxyribosides in the frog's egg. *Nature*, **169**: 245–246.

LØVTRUP, S., 1954. Chemical differentiation during amphibian embryogenesis. *Compt. rend. Lab. Carlsberg, Sér. chim.* (in press).

LÜTHI, H. R., 1938. Die Differenzierungsleistungen von Transplantaten der lethalen Bastardkombination *Triton* ♀ × *Salamandra* ♂. *Arch. f. Entw.*, **138**: 423–450.

MOORE, J. A., 1946. Studies in the development of frog hybrids I. Embryonic development in the cross *Rana pipiens* ♀ × *Rana sylvatica* ♂. *J. Exp. Zool.*, **101**: 173–220.

MOORE, J. A., 1947. Studies in the development of frog hybrids II. Competence of the gastrula ectoderm of *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrids. *J. Exp. Zool.*, **105**: 349–370.

MOORE, J. A., 1948. Studies in the development of frog hybrids III. Inductive ability of the dorsal lip region of *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrids. *J. Exp. Zool.*, **108**: 127–145.

SCHÖNMANN, W., 1938. Der diploide Bastard *Triton palmatus* ♀ × *Salamandra* ♂. *Arch. f. Entw.*, **138**: 345–375.

SHUMWAY, W., 1940. Normal stages in the development of *Rana pipiens*. *Anat. Rec.*, **78**: 130–147.

STEINERT, M., 1951. La synthèse de l'acide ribonucléique au cours du developpement embryonnaire des batraciens. *Bull. Soc. Chim. Biol.*, **33**: 549–554.

SZE, L. C., 1953. Changes in the amount of desoxyribonucleic acid in the development of *Rana pipiens*. *J. Exp. Zool.*, **12**: 577–602.

THE COMPOSITION OF THE BASIC PROTEINS OF ECHINODERM SPERM

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Protein with strongly basic properties has been found in the nuclei of most cells in association with desoxyribonucleic acid. In the sperm of some species of fish these proteins are simple in composition (protamines), containing only 7 or 8 amino acids and of these arginine accounts for about 90 per cent of the protein nitrogen (Hamer and Woodhouse, 1949; Felix, 1953). It has also been claimed that protamines are found in other animals, for example in fowl sperm (*Gallus domesticus*) (Fischer, 1954). However, in the sperm of cod (Stedman and Stedman, 1951) and mammals (Dallam and Thomas, 1953) proteins of histone type have been reported and these differ from protamines in having 16 amino acids. Arginine is again the principal component (20–30 per cent protein N) and, along with lysine and histidine, gives the protein its characteristic basic properties (Hamer, 1951, 1953a).

These basic proteins are believed to form some part of the structure of the chromosomes of the cells and so it is particularly interesting to know if they show species differences in composition. With this in view, basic proteins have been isolated from the spermatozoa of three echinoderms, the sea urchin, sand dollar and starfish, and the amino acid compositions determined. A similar protein isolated from the sperm of a mollusc, the squid, was also examined. The only previous chemical examination of the basic proteins of the sperm of invertebrates has been by Hultin (1947) and Hultin and Herne (1948). These workers reported that the compositions of sea urchin and mollusc basic sperm proteins were intermediate between protamines and histones, containing in all 11 amino acids. There was a considerable difference between the mollusc and sea urchin proteins while the basic proteins from different species of sea urchin were more similar in composition.

The results presented here show that in the species examined the sperm contain histone-type basic proteins and that there are significant species differences in composition. Two preliminary reports have included some of the results presented here (Hamer, 1953b, 1954).

Materials and Methods

Sperm from sea urchins (*Arbacia punctulata*) and sand dollars (*Echinarachnius parma*) were obtained by injection of 0.5 *M* KCl and the sperm collected in sea water. Ripe testes of starfish (*Asterias forbesi*) and squid (*Loligo pealii*) were removed, cut into small pieces and the sperm allowed to shed into sea water. The sperm were collected by centrifugation, washed first with sea water and then several times with a saline-citrate solution (0.14 *M* NaCl, 0.02 *M* citric acid).

Sperm nuclei so obtained were next extracted directly with 0.2 *M* HCl for 24 hours and then, after centrifuging, the extract was dialyzed against distilled water. All these operations were carried out in the cold. This extract now contained the protein or group of proteins classified as basic nuclear proteins (protamines and histones) since they are of nuclear origin, are extracted by acid, and remain soluble after removal of the acid by dialysis (Hamer, 1953a). It was found that the basic protein in the dialyzed solution could not be precipitated by addition of small amounts of ammonia (unlike mammalian histones) or sodium hydroxide and so picric acid was added and the protein picrate collected. This was then dissolved in acetone and any insoluble material centrifuged off. The solubility of basic protein picrates in acetone has previously been used for fractionation by

TABLE I

Composition of basic proteins

	Sea urchin*	Sea star	Sand dollar	Squid
Alanine	9.8	9.85	9.3	4.2
Ammonia	3.8	3.9	3.7	4.0
Arginine	28.0	23.8	34.1	47.7
Aspartic acid	2.7	2.9	1.9	3.4
Glutamic acid	3.9	4.0	3.0	3.7
Glycine	5.7	4.4	4.85	3.6
Histidine	2.1	2.9	1.0	2.0
Leucine	7.2	5.8	6.1	5.8
Lysine	17.1	19.0	13.9	8.2
Phenylalanine	1.0	0.9	0.9	1.0
Proline	4.4	4.25	3.4	2.6
Serine	4.0	3.8	5.3	4.8
Threonine	3.45	3.1	4.2	2.1
Tyrosine	1.1	1.1	1.1	1.9
Valine	3.7	3.9	4.2	2.8
Methionine	1.0	1.4	x	x

Amino acid nitrogen expressed as percentage of protein nitrogen.

* Cystine and tryptophane estimation were carried out only on the sea urchin basic protein. Neither was present in significant amount (less than 0.1 and 0.06%, respectively). The nitrogen content of this protein sulphate was 14.4 per cent.

x Not estimated.

Rasmussen (1934) and Hultin (1947). Sulphuric acid was added dropwise until precipitation of the protein sulphate was complete. The sulphate was dissolved in a small amount of water and re-precipitated by addition of acetone, to remove any remaining picric acid. After washing again with acetone the white protein sulphate was dried *in vacuo*. It may be noted here that similar techniques were applied for the preparation of basic fractions from echinoderm eggs following direct extraction of the eggs with acid. Small amounts of proteins were obtained but these were insufficient for quantitative analysis.

The analytical methods used were those previously applied to thymus histones (Hamer, 1951, 1953a). The protein was hydrolyzed for 18 hours with 6 N HCl under reflux. After repeated evaporation to remove acid, aliquots were analyzed by the starch column chromatographic technique of Stein and Moore (1949).

Separation of leucine and isoleucine was not carried out and these results are reported together as leucine. The identity of all components was confirmed separately by paper chromatography. Methionine, cystine and tryptophane were tested for independently when sufficient material was available by the following methods: methionine by the nitroprusside reaction (Horn, Jones and Blum, 1946); tryptophane using p-dimethylaminobenzaldehyde in sulphuric acid on unhydrolyzed protein (Spies and Chambers, 1949); cystine by Shinohara's modification of the Folin-Winterstein reaction (Block and Bolling, 1951). Nitrogen was determined by a micro-Kjeldahl method and phosphorus by Holman's (1943) colorimetric method after perchloric acid combustion. Recoveries of protein nitrogen in terms of amino acid nitrogen and ammonia ranged from 95–99 per cent.

Results

The results of the amino acid analyses are given in the table. It will be seen that all the basic proteins examined have the general type of composition found in the histone group. In each case, the basic amino acids arginine, lysine, and histidine together account for about 50 per cent of the protein nitrogen. Only small amounts of the aromatic amino acids tyrosine and phenylalanine are present and, in the case of the sea urchin specimen, analysis showed there was no significant amount of tryptophane or cystine. Glutamic and aspartic acids represent 7–8 per cent of the whole. Compared with histones from mammalian cells, the echinoderm sperm histones contain appreciably more alanine and lysine but less leucine, phenylalanine and tyrosine. The protein from the squid sperm contains considerably more arginine than is found in the specimens from sea urchin and starfish by colorimetric estimation though this was not sufficient to be detected chromatographically on the starch columns.

From estimations of the nitrogen and phosphorus contents of sea urchin sperm nuclei before and after extraction with acid it was estimated that about 27 per cent of the dry weight of the nuclei was nucleic acid, 21 per cent histone extractable by acid and the remaining 52 per cent acid-insoluble protein. The nature of this protein has not been investigated though in the case of mammalian tissues it has been found to be similar in composition to the basic protein but to contain, in addition, measurable amounts of cystine and tryptophane.

Discussion

The results presented above differ from those of Hultin and Herne (1948) in that 16 amino acids were found in the basic proteins of all the species examined whereas only 11 had previously been reported for related species (*e.g., Arbacia lixula, Brissopsis lyrifera*). It would seem unlikely that this is due to a species difference but is more likely to arise from differences in analytical methods. For qualitative analysis Hultin and Herne carried out a preliminary fractionation of the hydrolysates on carbon and other adsorbents. Possibly the small amounts of tyrosine, phenylalanine, and methionine present were lost in this treatment but the failure to detect glutamic acid and aspartic acid is difficult to account for.

Significant species differences were found between the three echinoderms examined. Considerable variations occur in the lysine and arginine contents while there are minor variations in the amounts of other amino acids, for example

leucine and glycine. The molluscs, as represented by the squid specimen, show more considerable changes in composition but the basic protein is still of the histone-type. At the moment there seems no rational way of predicting whether the sperm nuclei of a particular species will contain a protamine or a histone as the basic component of the desoxyribonucleo-protein complex. A number of fish and fowl sperm contain protamines while the sperm of some mammals and invertebrates contain histones. At present there are insufficient data to justify a claim that protamines occur widely in sperm nuclei (Fischer, 1954).

The results quoted for the relative amounts of histone, nucleic acid and acid-insoluble material are in agreement with those obtained by Bernstein and Mazia (1953) using sperm nucleoprotein of the sea urchin *Strongylocentrotus purpuratus*. There is certainly a considerable amount of protein associated with the nucleic acid in the sperm nuclei which is not "histone" in the sense that it is not readily extracted by acid. This has also been found in studies on mammalian somatic cells, and in discussing the possible arrangement of these fractions it has been suggested that the histone may be held by simple ionic forces while at least part of the acid-insoluble protein is held by chemical links to the nucleic acid (Hamer, 1953a, 1954). The work of Barton (1952a, 1952b) on the fractionation of invertebrate sperm with acid and nucleases lends some support to such a structural arrangement.

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Summary

1. Basic proteins have been extracted from sperm of the sea urchin, sand dollar and starfish, and also from the squid. The amino acid composition of these proteins has been determined.

2. All the basic proteins were of histone type and were similar in general properties and composition to mammalian histones.

3. There are significant species differences among the echinoderms examined, particularly in the arginine and lysine contents. The basic protein of squid sperm contains much more arginine and less lysine than the echinoderm specimens.

LITERATURE CITED

Barton, J., II, 1952a. Nucleoprotein complexes of sperm nuclei. *Biol. Bull.*, **103**: 314.

Barton, J., II, 1952b. Action of DN-ase on cell nuclei. *Biol. Bull.*, **103**: 319–327.

Bernstein, M. H., and D. Mazia, 1953. Desoxyribonucleoprotein of sea urchin sperm. *Biochim. Biophys. Acta*, **10**: 600–606.

Block, R. J., and D. Bolling, 1951. Amino acid composition of proteins and foods. 2nd. ed. Charles C. Thomas. Springfield.

Dallam, R. D., and L. E. Thomas, 1953. Chemical studies on mammalian sperm. *Biochim. Biophys. Acta*, **11**: 79–89.

Felix, K., 1953. Protamines and nucleoprotamines. In "Chemical Structure of Proteins." J. and A. Churchill: London.

Fischer, H., 1954. Nucleoprotamines. *Trans. Farad. Soc.*, **50**: 295.

Hamer, D., and D. L. Woodhouse, 1949. Amino-acid composition of salmine. *Nature*, **163**: 689–690.

HAMER, D., 1951. Aspects of the chemistry of proteins in the nucleus. *Brit. J. Cancer*, **5**: 130–139.

HAMER, D., 1953a. A comparison of different protein fractions obtained from thymus nuclei. *Brit. J. Cancer*, **7**: 151–156.

HAMER, D., 1953b. Isolation of protein fractions from echinoderm sperm and eggs. *Biol. Bull.*, **105**: 389–390.

HAMER, D., 1954. Composition and interactions of nucleoproteins. *Trans. Farad. Soc.*, **50**: 297.

HOLMAN, W. I. M., 1943. New technique for the determination of phosphorus by the molybdenum blue method. *Biochem. J.*, **37**: 256–259.

HORN, M. J., D. B. JONES AND A. E. BLUM, 1946. Colorimetric determination of methionine in proteins and foods. *J. Biol. Chem.*, **166**: 313–320.

HULTIN, 1947. Physiological effects of basic sperm proteins. *Arkiv. Kemi, Mineral., Geol.*, **24B**; No. 12. 1–6.

HULTIN, T., AND R. HERNE, 1948. Amino acid analysis of a basic protein fraction from sperm nuclei of some different invertebrates. *Arkiv. f. Kemi, Mineral., Geol.*, **26A**; No. 20. 1–8.

MOORE, S., AND W. H. STEIN, 1949. Chromatography of amino acids on starch columns. *J. Biol. Chem.*, **178**: 53–77.

RASMUSSEN, K. E., 1934. Darstellung und Fraktionierung von Clupein. *Hoppe-Seyl. Zeitschr.*, **224**: 97–115.

SPIES, J. R., AND D. C. CHAMBERS, 1949. Chemical determination of tryptophane in proteins. *Anal. Chem.*, **21**: 1249–1266.

STEDMAN, E., AND E. STEDMAN, 1951. Basic proteins of cell nuclei. *Phil. Trans. Roy. Soc.*, **B235**: 565–595.

THE RELATIONSHIP BETWEEN CORPORA ALLATA AND REPRODUCTIVE ORGANS IN STARVED FEMALE LEUCOPHAEA MADERAE (BLATTARIA)

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It has long been known that hormonal regulation plays an important role in insect reproduction. Aside from the medial neurosecretory cells of the protocerebrum whose action on egg development has been demonstrated in Calliphora (Thomsen, 1952), the corpora allata are necessary for the normal function of the ovary and the accessory sex glands of most insect species investigated (see review: Scharrer, 1953). In the absence of the corpora allata, the oocytes remain in an immature state characteristic of the onset of a given reproductive cycle, and the accessory sex glands stay inactive.

Another important factor in insect reproduction is nutrition. Egg production does not occur in many insect species, when food in adequate quantity and quality is lacking. The question arises, whether the inability of starved insect females to reproduce mature ova constitutes a primary effect, or whether it is due to an influence of inanition on the endocrine system. A previous study indicated that the latter alternative applies to the activity of the corpus allatum of the milkweed bug, *Oncopeltus fasciatus* (Johansson, 1954). In the present paper this problem is studied in a different species belonging to another order of insects.

Material and Methods

Leucophaea maderae is an ovoviviparous cockroach which has been bred in the laboratory for years (*cf.* Scharrer, 1946a, 1946b). Freshly emerged adults which were to serve as donors and hosts, were taken from the stock colonies and isolated in glass jars of pint size. The food consisted of dogchow and apples. The specimens which were used as hosts were starved from the day of emergence and only given water. All specimens were kept at room temperature, *i.e.*, at 26–33° C.

Besides Leucophaea, also females of *Oncopeltus fasciatus*, were used as donors. They were taken from a laboratory colony fed on dried seeds of the milkweed.

Organs to be implanted were dissected out in insect Ringer, taken up in a fine glass capillary connected with a plunger device, and implanted into the abdomen of the host.

The corpora allata of Leucophaea are paired organs. The implantation of one corpus allatum, therefore, means that only half of the organ present in a single individual was used. When three or four corpora allata were implanted, they were taken from two individuals of the same sex and adult age. In Oncopeltus

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the corpus allatum is unpaired. Thus the number of corpora allata implanted in these cases is equivalent to the number of specimens used as donors. Abdominal ganglia of Leucophaea were implanted as controls.

The hosts were killed at various intervals after the implantation and examined. In a few cases they died before examination took place.

Results

In newly emerged females of Leucophaea the ovaries are small, the longest oocytes being about 1 to 1.4 mm in length. No yolk is present. The accessory

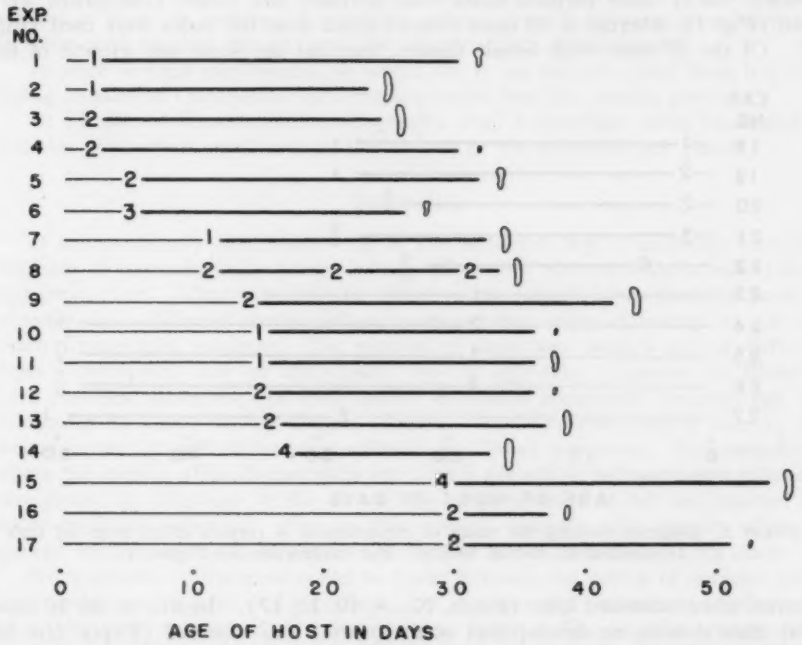

FIGURE 1. Diagram showing the result of implantations of corpora allata from fed females of *Leucophaea maderae* to starved female hosts. The number of corpora allata implanted and the day of implantation are given in the breaks of the lines. The right end of the heavy lines marks the day on which the host was examined. A schematic drawing in mm. of the largest oocyte observed is shown on the right.

sex glands are inconspicuous and do not show signs of secretion (Scharrer, 1946b). In fed females the oocytes grow and reach their maximal size of about 5 mm. after four to five weeks. During this period yolk is deposited in the oocytes. The accessory glands increase in size and obtain a bluish color due to the accumulation of their secretory product. The corpora allata are necessary, both for the growth of the oocytes and for the secretory activity of the accessory sex glands (Scharrer,

1946b). The corpora allata of Leucophaea do not show any appreciable increase in size during this period of activity.

Starvation of the females of this species prevents egg maturation (Scharrer, 1943). The accessory glands may show some secretory activity, but this does not seem to reach the level characteristic of normally fed specimens. Starved animals could be kept alive up to 74 days, but in none of the 16 specimens examined had the oocytes grown, and no yolk was deposited.

Starvation does not seem to influence the size of the corpora allata of Leucophaea.

Altogether 27 experiments were carried out in which Leucophaea served as donors. In 17 cases corpora allata from normally fed female Leucophaea were used (Fig. 1), whereas in 10 cases corpora allata from fed males were used (Fig. 2). Of the 17 cases with female donors, four did not show any growth of the

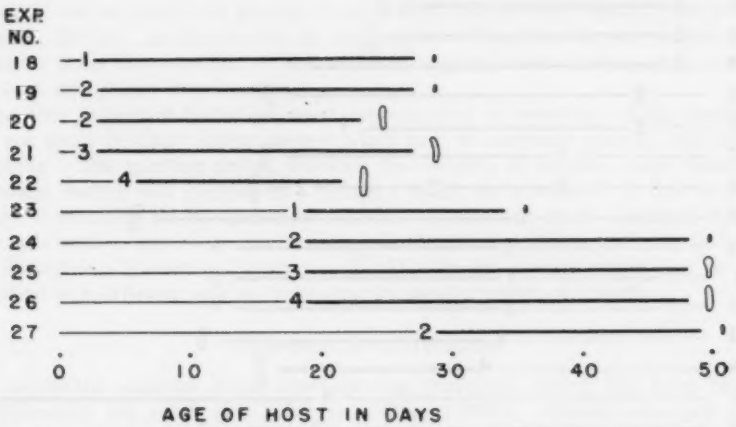

FIGURE 2. Diagram showing the result of implantations of corpora allata from fed male Leucophaea to starved females. For explanation see Figure 1.

ovaries when examined later (Expts. No. 4, 10, 12, 17). In five of the 10 cases with male donors, no development of the ovaries was observed (Expts. No. 18, 19, 23, 24, 27). In the rest of these experiments the implantation of corpora allata into starved hosts resulted in deposition of yolk and growth of the oocytes. As to the negative results, it ought to be mentioned that even in fed females sometimes no eggs develop. The number of grown oocytes observed in these implantation experiments ranged from one (Expts. No. 6, 7) up to normal numbers (Expts. No. 16, 26), *i.e.*, up to 40 (Scharrer, 1946b). In a few cases the shape of the grown oocytes was somewhat irregular. In two cases (Expts. No. 14, 22) eggs were deposited. Premature deposition of eggs is occasionally observed in normal females under laboratory conditions.

The adult age of female donors ranged from one (Expts. No. 7, 13) up to 54 days (Expt. No. 12). In the majority of the cases, the females were from 18 to 38 days old; nearly all had growing oocytes. The female which was 54

days old, had already deposited her eggs when used as a donor. The age of the male donors ranged from 18 to 35 days. It is seen that in the case of female donors even one single corpus allatum from a one-day old specimen can give positive results (Expt. No. 7). It seems that when males are used as donors, two or three corpora allata are necessary to be effective.

Growth of the oocytes took place even when the host had starved up to 30 days before implantation (Expt. No. 16).

The accessory sex glands also responded to the corpus allatum implants. In the experiments in which no growth of the oocytes took place, the accessory sex glands did not seem to increase their activity beyond a level which may be observed also in starved females. In cases with grown oocytes, the accessory sex glands seemed to show an increase in secretory activity beyond the starvation level, and in some cases reached the maximal size characteristic of fed females.

In none of eight experiments, in which one to six corpora allata from fed, egg-laying females of Oncopeltus had been implanted, had the oocytes grown.

In 12 starved females abdominal ganglia from Leucophaea were implanted as controls. No effect was found on the oocytes or the accessory sex glands.

Discussion

It has previously been found that in the milkweed bug, *Oncopeltus fasciatus* implants of physiologically active corpora allata into starved females may induce egg production (Johansson, 1954). The present paper shows that the same is true also in a different species belonging to another order of insects, *i.e.*, in the roach *Leucophaea maderae*. The ovaries of these two species are of different types. Oncopeltus has an acrotrophic type of egg tube, whereas the Blattaria have panoistic ovarioles. In both species starvation apparently prevents egg development by acting on the corpus allatum. In Oncopeltus this is seen in the lack of normal growth of the corpus allatum in starved specimens. In Leucophaea, where the corpora allata do not show any visible growth in fed specimens following emergence, no difference in the size of the corpora allata of fed and starved females could be observed. Also in this species, however, the corpora allata of starved females appear to be physiologically less active than those of fed ones.

No qualitative differences could be found between the action of corpora allata from males and females of Leucophaea, but it seems that quantitative differences exist, the male corpora allata being the less active. The negative results with implants from Oncopeltus may also have a quantitative rather than a qualitative explanation.

The presence of corpora allata has been found necessary for the normal activity of the accessory sex glands. In allatectomized females of Leucophaea the accessory sex glands do not secrete (Scharrer, 1946b). Since these glands in starved females sometimes show a limited secretory activity, it would seem that they have a lower threshold of response to the corpus allatum hormone than the ovaries, and that starvation does not totally prevent the secretion of corpus allatum hormone. A similar phenomenon is observed in Melanoplus where secretion in the oviduct may take place under conditions which prevent yolk production, and Pfeiffer (1945) suggests that the oviducts are able to respond to the corpus allatum hormone before the ovaries react with yolk production.

The author wishes to express his gratitude to Dr. B. Scharrer for supplying the material of Leucophaea and for helpful suggestions.

Summary

1. In starved females of *Leucophaea maderae* whose ovaries remain immature, egg development can be induced by the implantation of corpora allata from fed donors. Implants from adult males have the same qualitative effect as those from females, but they are by comparison less active. Thus in Leucophaea as well as in Oncopeltus (Johansson, 1954), the inhibition of ovarian development caused by starvation is not a direct effect, but is mediated through the corpora allata.

2. While the oocytes of female Leucophaea, starved from emergence, remain undeveloped, their accessory sex glands may show some measure of secretory activity. The result indicates that these glands whose activity depends on the corpus allatum have a lower threshold of response than the ovaries, and that starvation does not totally prevent the secretion of corpus allatum hormone.

LITERATURE CITED

Johansson, A. S., 1954. Corpus allatum and egg production in starved milkweed bugs. *Nature*, **174**: 89.

Pfeiffer, I. W., 1945. Effect of the corpora allata on the metabolism of adult female grasshoppers. *J. Exp. Zool.*, **99**: 183–233.

Scharrer, B., 1943. The influence of the corpora allata on egg development in an orthopteran (*Leucophaea maderae*). *Anat. Rec.*, **87**: 471.

Scharrer, B., 1946a. The role of the corpora allata in the development of *Leucophaea maderae* (Orthoptera). *Endocrinol.*, **38**: 35–45.

Scharrer, B., 1946b. The relationship between corpora allata and reproductive organs in adult *Leucophaea maderae* (Orthoptera). *Endocrinol.*, **38**: 46–55.

Scharrer, B., 1953. Comparative physiology of invertebrate endocrines. *Ann. Rev. Physiol.*, **15**: 457–472.

Thomsen, E., 1952. Functional significance of the neurosecretory brain cells and the corpus cardiacum in the female blow-fly, *Calliphora erythrocephala* Meig. *J. Exp. Biol.*, **29**: 137–172.

ADENOSINETRIPHOSPHATASE ACTIVITY OF AMERICAN COCKROACH [1] AND WOODROACH [2] THORACIC MUSCLE [3]

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The enzymes of mammalian tissues, particularly muscle and liver, which split inorganic phosphate from adenosinetriphosphate (ATP) have received considerable study during the past several years. Du Bois and Potter (1943) developed a method for the quantitative determination of the adenosinetriphosphatase (ATPase) of tissue homogenates and showed that it was greater in cardiac and skeletal muscles than in liver, lung, kidney, submaxillary gland, spleen, pancreas, brain, and smooth muscle of the normal rat. The ATPase in the corpora lutea of rat ovaries changes little during pregnancy and lactation (Biddulph *et al.*, 1946). More recent studies have been concerned with the kinds of ATPase found in liver and the particles with which it is associated. Kielley and Kielley (1951, 1953) and Novikoff *et al.* (1952) showed that liver mitochondria dephosphorylate ATP. A "soluble" ATPase was obtained from muscle (Kielley and Meyerhof, 1948), and from the supernatant fraction of liver (Swanson, 1951). The ATPase of yeast has been purified by Meyerhof and Ohlmeyer (1952), and that of liver by Swanson (1952). Heppel and Hilmoe (1953) separated and partially purified three different ATPases from bull seminal plasma and studied their mechanism of action on ATP. The ATPase of extracts of rabbit muscle was studied by Humphrey and Humphrey (1950). The kinetics of the dephosphorylation of ATP by rabbit myosin was investigated by Ouellet, Laidler and Morales (1952), and the mechanism of hydrolysis of ATP by lobster muscle was studied by Koshland and Clarke (1953).

The presence of ATP in the tissues of *Drosophila melanogaster* was reported by Albaum and Kletzkin (1948), and in insect muscle by Calaby (1951). The major part of the work on the enzymatic dephosphorylation of ATP by insect tissues has been done with insect muscle. Gilmour (1948) found a soluble Mg-activated enzyme in myosin extracts of grasshopper muscle which split both high energy phosphate bonds of ATP. In a further study of muscle of locusts (*Locusta migratoria* and *Castrimargus musicus*) Gilmour and Calaby (1952) demonstrated that myokinase or adenylate kinase was not responsible for the removal of the second phosphate from ATP and that the apyrase could utilize adenosine di-

[1] *Periplaneta americana.*

[2] *Leucophaea maderae.* In a previous paper the common name "woodroach" was used for this species, following the usage suggested by Scharrer (1951). Taxonomists, however, generally use the common name of "Madeira roach" for this species, as pointed out by Gurney (1953), in order to avoid confusion with native species of the genus *Parablatta*, also referred to commonly as "woodroaches."

[3] This investigation was supported in part by a grant from funds supplied by the Wisconsin Alumni Research Foundation.

phosphate (ADP), inosine triphosphate (ITP), and inosine diphosphate (IDP) as substrates. Sacktor (1953) found that the ATPase of mitochondria from the thoraces of the house fly, *Musca domestica*, is activated by Mg and Mn but not by Ca ions. A Ca-activated ATPase was found in the muscle fibrils, and adenylate kinase was present in the mitochondria. Sacktor *et al.* (1953) studied the dephosphorylation of ATP by several tissues of the American cockroach, *Periplaneta americana*. These tissues were rated in the following order of decreasing activity: muscle, fat body, Malphighian tubes, nerve cord, brain, hindgut, foregut, and midgut. Mg was more effective than Ca ions in activating the ATPase of these tissues. The activity of the muscle and hindgut of the female was greater than that in these tissues of the male.

Since the catalytic breakdown of ATP by ATPase presumably supplies energy for cellular functions, it was of interest to study the ATPase system in the thoracic muscle of the American cockroach, *Periplaneta americana*, and to determine the relation of the activity of this enzyme to the age and sex of the roach. Results of preliminary studies of the thoracic muscle of the woodroach, *Leucophaea maderae*, are also reported.

Experimental Materials and Methods

The chemicals used were reagent grade and the cofactors and substrates were obtained from different sources.[4] The sodium salts of these compounds were adjusted to pH 7.4 for experimental purposes.

The roaches from which tissue was obtained were isolated at one day of age so that insects of known ages would be available for experimental purposes. A few experiments were done, however, with tissue from insects of unknown ages. The roaches were kept in glass jars and were given water and food at regular intervals.

The meso- and metathoracic muscles were removed immediately after the roaches were killed. The technique of dissecting out these tissues was described in detail in a previous paper (McShan, Kramer and Schlegel, 1954). The muscle was placed in a sharp-pointed glass homogenizer in an ice bath and homogenized in sufficient water to give a one per cent homogenate (weight of tissue in grams multiplied by 99 gives the required ml. of water).

The ATPase activity of the muscle homogenates was determined by the method reported by DuBois and Potter (1943). Three amounts of tissue were used in each determination. The controls used were the complete system without tissue, and without ATP, enabling us to make correction for the inorganic phosphate present in the reaction medium, in addition to that released by the action of the ATPase.

The constituents of the system were measured into small tubes and heated to

[4] Adenosinetriphosphate (ATP), adenosinediphosphate (ADP) and adenosine-5′-phosphate (AMP) were obtained from the Pabst Laboratories, Milwaukee, Wisconsin; glucose-1-phosphate, fructose-1-phosphate and fructose-1,6-disphosphate from the Schwartz Laboratories, Inc., New York; sodium beta-glycerophosphate and sodium phosphoglycerate from National Biochemicals, Inc., Chagrin Falls, Ohio; disodium phenylphosphate and sodium phenolphthalein phosphate from Paul-Lewis Laboratories, Inc., Milwaukee, Wisconsin; paranitrophenylphosphate from the Sigma Chemical Company, St. Louis, Missouri; and a sample of sodium phenolphosphate purified in our laboratory.

38° C. in a water bath. The proper amounts of tissue were then added to the tubes and incubation was continued for 15 minutes at which time the reaction was stopped by addition of 0.2 ml. of 50 per cent cold trichloracetic acid (TCA). The tubes were centrifuged to remove the precipitate and the amount of inorganic phosphorus in 0.3 ml. of the supernatant was determined by the method of Fiske and Subbarrow (1925) as a measure of the ATPase activity of the muscle

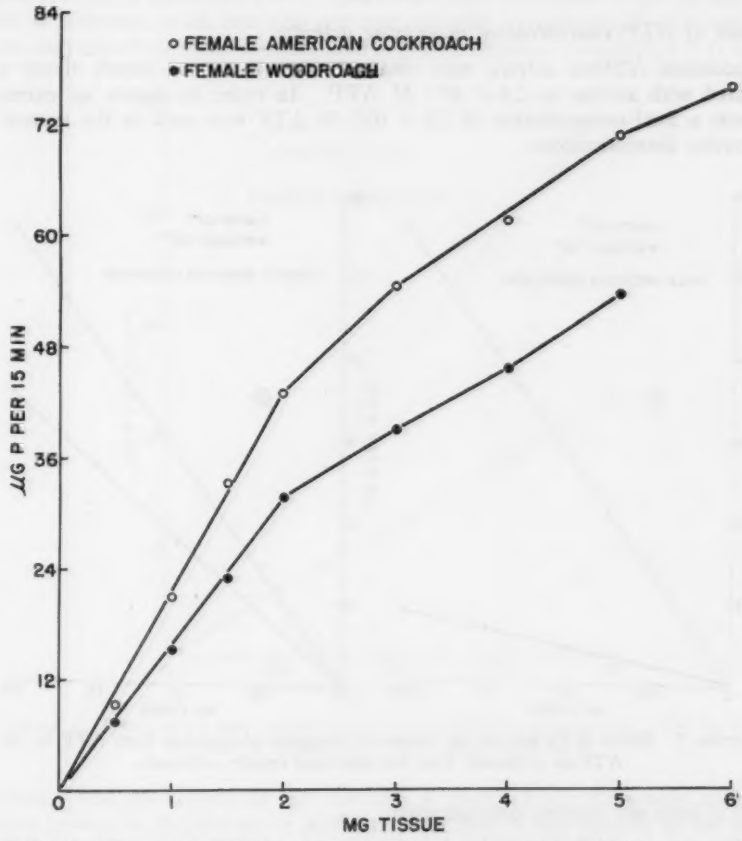

FIGURE 1. Curves showing the relations between the μg. of inorganic phosphorus released from 2.8×10^{-3} *M* ATP by different amounts of muscle tissue. The relation is linear with 0.5 mg. to 2.0 mg. of fresh tissue.

homogenate. Known amounts of phosphorus in the form of KH_2PO_4 were run with each determination as standards to serve as a basis for calculating the phosphorus content of the TCA supernatants.

One unit of ATPase activity is defined as the amount of enzyme required to release 1 μg. of inorganic phosphorus from ATP in 15 minutes at 37° C. (Du Bois

and Potter, 1943). The μg. of phosphorus released in 15 minutes by the different amounts of tissue were used for calculating the μg. of phosphorus hydrolyzed by 1 mg. of tissue. The values used for preparing the graphs and given in the tables are expressed in terms of the μg. of phosphorus released by 1 mg. of muscle in 15 minutes. These values represent the number of ATPase units per mg. of muscle.

Results

Relation of ATP concentration to enzyme activity

Maximum ATPase activity was obtained when 2 mg. of muscle tissue were incubated with as low as 1.4×10^{-3} *M* ATP. In order to insure an excess of substrate a final concentration of 2.8×10^{-3} *M* ATP was used in the system for the routine determinations.

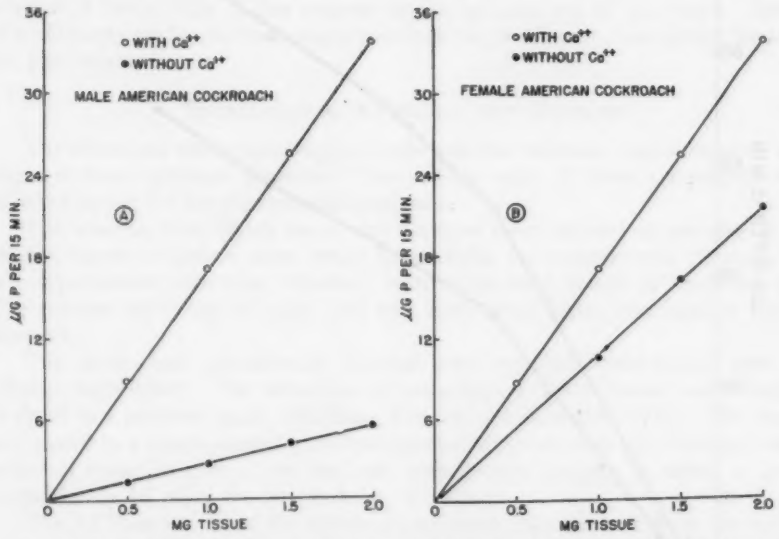

Figure 2. Effect of Ca ions on the release of inorganic phosphorus from ATP by the ATPase of muscle from the male and female cockroach.

Effect of time and enzyme concentration

The rate at which inorganic phosphorus was released from ATP by different amounts of tissue was not constant with time. This decrease in ATPase activity with time has been reported for liver homogenates (Novikoff *et al.*, 1952), for purified yeast ATPase (Meyerhof and Ohlmeyer, 1952), and for mitochondria of flies (Sacktor, 1953).

The results given graphically in Figure 1 for the muscle of the female cockroach and female woodroach show that the amounts of inorganic phosphorus released were linearly related to the enzyme concentration when 0.5, 1.0, 1.5 and 2.0 mg. of

tissue were used. This linear relation was obtained when the incubations were continued for 10, 20 and 30 minutes.

Effect of activating ions

Bivalent cations such as Ca, Mg and Mn are known to activate the ATPase of mammalian, yeast and certain insect preparations. Results represented in Figure 2A and B show that the ATPase in the thoracic muscle of the female cockroach is activated much less (58 per cent increase in activity) by Ca ions than is the enzyme of the male muscle (533 per cent increase in activity). It is also

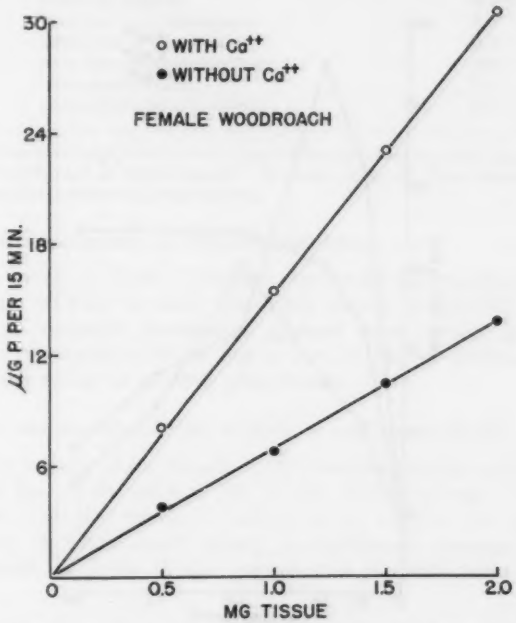

FIGURE 3. Effect of Ca ions on the release of inorganic phosphorus from ATP by the ATPase of muscle from the female woodroach.

of interest that the activity of the ATPase of muscle from the female cockroach is much greater in the absence of added Ca ions than is this enzyme in the muscle of the male. Sacktor (1953) reported results of this kind for the muscle and hindgut of male and female cockroaches. In the light of these results the question arises as to whether the greater ATPase activity of the female muscle without added Ca ions may not be due to a greater concentration of these ions in this muscle than in the muscle of the male roach.

The activation of the ATPase of muscle from the female woodroach is shown in Figure 3. The degree of Ca activation in this case is less than was found for the male but greater than that obtained for the female American cockroach.

As has been found for the ATPase of other kinds of tissue Mg and Mn ions were also found to activate the ATPase of muscle from the male and female cockroach. The degree of activation with different concentrations of Ca, Mg and Mn ions is shown in Figure 4. The ATPase activity was more nearly the same when the concentration of each of the ions was 2.8×10^{-3} *M*. Mg ions were more effective in activating the system than Ca and Mn ions at a concentration of 1.4×10^{-3} *M*. Similarly, Sacktor (1953) found that Mg ions were more effective than Ca ions at a concentration of 1×10^{-3} *M*. Ca ions were more effective in activating the ATPase of cockroach muscle at concentrations greater than 2.8×10^{-3} *M*.

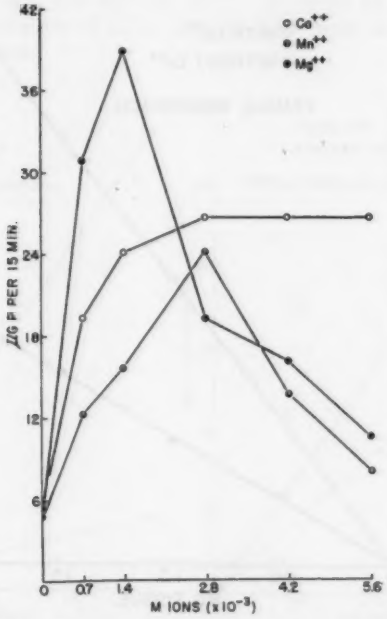

FIGURE 4. Activation of the ATPase of cockroach muscle by increasing concentrations of Ca, Mn and Mg ions.

On the basis of the above results 0.05 ml. of 0.04 *M* $CaCl_2 \cdot 2H_2O$ and 0.2 ml. of 0.01 *M* ATP (2.8×10^{-3} *M* final concentration of each) were used in the system for the determination of the ATPase of cockroach and woodroach muscle. The amounts of the other reagents used in the system were: 0.15 ml. of 0.5 *M* sodium diethylbarbiturate buffer of pH 7.4 and, as indicated above, 0.05, 0.10 and 0.15 ml. of one per cent homogenate plus sufficient water to give a total volume of 0.7 ml. for incubation. Three amounts of tissue were used for each determination to demonstrate that the phosphate released from the ATP was linearly related to the ATPase of these amounts of tissue.

TABLE I

Comparison of ATP and other phosphate esters as substrates for ATPase of male American cockroach muscle

Substrate*	ATPase Units**
Adenosinetriphosphate	15.1
Adenosinediphosphate	0.6
Fructose-6-phosphate	1.2
Glucose-1-phosphate	0.6
beta-Glycerophosphate	0.0
Phenylphosphate	0.9
Fructose-1,6-diphosphate	0.0
Adenosine-5′-phosphate	0.0
para-Nitrophenylphosphate	0.0
Phosphoglycerate	0.0
Phenolphthaleinphosphate	0.9

* The substrates were used in the form of the sodium salts and in a final concentration 0.003 *M*.
** Units per milligram of fresh muscle. Average value of three experiments using three different amounts of tissue for each experiment.

Effect of muscle homogenate on different substrates

The results given in Table I indicate that under the conditions used in these experiments the ATPase of male cockroach muscle is specific for ATP. The small amount of inorganic phosphorus released from certain of the other compounds used as substrates might be due to low activity of ATPase for these compounds, or to the action of another phosphatase.

Relation of age and sex to ATPase activity of cockroach muscle

The ATPase activity of the muscle of the American female cockroach appears to be greater than that of the male at 10, 20 and 30 days of age (Table II). This is in agreement with the results of Sacktor *et al.* (1953) who reported that the ATPase activity of female roach muscle is significantly greater than that of the male. The results of Table II also suggest that for both sexes there is a trend

TABLE II

Adenosinetriphosphatase of thoracic muscle from the American cockroach

Sex	Age, days	ATPase units*
Male	10	19.2
	20	16.9
	30	15.7
Female	10	21.6
	20	19.5
	30	18.1

* Units per milligram of fresh tissue. The values are averages of two to five determinations in which three different amounts of tissue were used. The time of incubation was 15 minutes. Ca ions were present in a concentration of 2.8×10^{-3} *M*.

toward a decrease in ATPase activity with an increase in age from 10 to 30 days. A definite conclusion on this age-ATPase activity relation is not justified, however, until determinations are made using a larger number of roaches over a wider age range.

Results of preliminary experiments on the thoracic muscle of the woodroach of various ages suggest that the sex difference in ATPase activity of the American cockroach does not exist in the woodroach, *Leucophaea maderae*. In this connection it should be mentioned that the succinoxidase activity of muscle from male woodroaches is essentially the same as the activity of muscle from the female, whereas the activity of this system in male American cockroach muscle is three to four times that of the female.

Lawrie (1952), in a study of the biochemical differences of red (high in myoglobin content) and white skeletal muscle (low in myoglobin content) of various vertebrates, indicated that in general high myoglobin content in muscle is associated with high enzymatic activity for succinic dehydrogenase, succinoxidase and cytochrome oxidase. ATPase activity, on the other hand, decreases with an increase in myoglobin content. It is interesting to note that the male American cockroach has red thoracic muscles and shows higher succinoxidase activity and lower ATPase activity than these muscles in the female which are white. There is no evidence for the presence of myoglobin in insect muscle. Sacktor *et al.* (1953), however, have suggested that in insects the difference in color may be due to the cytochrome content. Whatever the color difference may be due to, it should be noted that in the woodroach, *Leucophaea maderae*, in which both sexes have red muscles, succinoxidase and ATPase activity are apparently equivalent in both sexes.

Summary

1. The adenosinetriphosphatase (ATPase) activity of the thoracic muscle of the American cockroach, *Periplaneta americana*, and the woodroach, *Leucophaea maderae*, was studied. The optimum conditions for eliciting maximum ATPase activity of the muscle of these two species are: 0.2 ml. of 0.01 *M* ATP, 0.05 ml. of 0.04 *M* $CaCl_2 \cdot 2H_2O$, 0.15 ml. of 0.5 *M* diethylbarbiturate buffer of pH 7.4, and 0.05, 0.10 and 0.15 ml. of one per cent homogenate.

2. The ATPase activity of the muscle from females was activated less by calcium than was that of the male American cockroach. The degree of calcium activation of the ATPase of the female woodroach muscle was intermediate between that of the male and female cockroach muscle.

3. The ATPase activity of muscle from female American cockroaches 10 to 30 days of age was greater than that of male roaches of the same ages. Present results suggest that there is not a difference in the ATPase activity of muscle from male and female woodroaches.

LITERATURE CITED

ALBAUM, H. G., AND M. KLETZKIN, 1948. Adenosinetriphosphate from *Drosophila melanogaster*. *Arch. Biochem.*, **16**: 333–337.

BIDDULPH, C., R. K. MEYER AND W. H. McSHAN, 1946. Adenosinetriphosphatase activity of lutein and ovarian tissues and weight of corpora lutea during the reproductive cycle of the rat. *Endocrinology*, **38**: 358–367.

CALABY, J. H., 1951. Adenosinetriphosphate from insect muscle. *Arch. Biochem. and Biophysics,* **31**: 294–299.

DUBOIS, K. P., AND V. R. POTTER, 1943. The assay of animal tissues for respiratory enzymes. III. Adenosinetriphosphatase. *J. Biol. Chem.,* **150**: 185–195.

FISKE, C. H., AND Y. SUBBAROW, 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.,* **66**: 375–400.

GILMOUR, D., 1948. Myosin and adenylpyrophosphatase in insect muscle. *J. Biol. Chem.,* **175**: 477–478.

GILMOUR, D., AND J. H. CALABY, 1952. The magnesium-activated apyrase of insect muscle. *Arch. Biochem. and Biophysics,* **41**: 83–103.

GURNEY, A. B., 1953. Distribution, general bionomies, and recognition characters of two cockroaches recently established in the United States. *Proc. U. S. Nat. Mus.,* **103**: 39–56.

HEPPEL, L. A., AND R. J. HILMOE, 1953. Mechanism of hydrolysis of adenosinetriphosphate. *J. Biol. Chem.,* **202**: 217–226.

HUMPHREY, B. A., AND G. F. HUMPHREY, 1950. The breakdown of adenosinetriphosphate in extracts of rabbit muscle. *Biochem. J.,* **47**: 238–244.

KIELLEY, W. W., AND O. MEYERHOF, 1948. Studies on adenosinetriphosphatase of muscle. II. A new magnesium-activated adenosinetriphosphatase. *J. Biol. Chem.,* **176**: 591–601.

KIELLEY, W. W., AND R. K. KIELLEY, 1951. Myokinase and adenosinetriphosphatase in oxidative phosphorylation. *J. Biol. Chem.,* **191**: 485–500.

KIELLEY, W. W., AND R. K. KIELLEY, 1953. A specific adenosinetriphosphatase of liver mitochondria. *J. Biol. Chem.,* **200**: 213–221.

KOSHLAND, D. E., AND E. CLARKE, 1953. Mechanism of hydrolysis of adenosinetriphosphate catalyzed by lobster muscle. *J. Biol. Chem.,* **205**: 917–924.

LAWRIE, R. A., 1952. Biochemical differences between red and white muscle. *Nature,* **170**: 122–123.

MCSHAN, W. H., S. KRAMER AND V. SCHLEGEL, 1954. Oxidative enzymes in the thoracic muscles of the woodroach, *Leucophaea maderae. Biol. Bull.,* **106**: 341–352.

MEYERHOF, O., AND P. OHLMEYER, 1952. Purification of adenosinetriphosphatase of yeast. *J. Biol. Chem.,* **195**: 11–17.

NOVIKOFF, A. B., L. HECHT, E. PODBER AND J. RYAN, 1952. Phosphatases of liver. I. The dephosphorylation of adenosinetriphosphate. *J. Biol. Chem.,* **194**: 153–170.

OUELLET, L., K. J. LAIDLER AND M. F. MORALES, 1952. Molecular kinetics of muscle adenosinetriphosphatase. *Arch. Biochem. and Biophysics,* **39**: 37–50.

SACKTOR, B., 1953. Investigations on the mitochondria of the house fly, *Musca domestica.* I. Adenosinetriphosphatases. *J. Gen. Physiol.,* **36**: 371–387.

SACKTOR, B., G. M. THOMAS, J. C. MOSER AND D. I. BLOCH, 1953. Dephosphorylation of adenosinetriphosphate by tissues of the American cockroach, *Periplaneta americana. Biol. Bull.,* **105**: 166–173.

SCHARRER, B., 1951. The woodroach. *Sci. Amer.,* **185**: 59–62.

SWANSON, M. A., 1951. Phosphatases of the liver. II. The so-called adenosinetriphosphatases. *J. Biol. Chem.,* **191**: 577–590.

SWANSON, M. A., 1952. Phosphatases of the liver. III. "Neutral" pyrophosphatase. *J. Biol. Chem.,* **194**: 685–693.

THE EFFECT OF COLCHICINE ON RECONSTITUTIONAL DEVELOPMENT IN DUGESIA DOROTOCEPHALA[1]

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Many investigators have used the regeneration phenomenon observed in planarians in an attempt to understand the basic mechanisms which control developmental processes. Most of these studies have been made at the organismic level. However, as early as 1904, Bardeen and Baetjer demonstrated that the regenerative powers of *Planaria maculata* and *P. lugubris* could be destroyed by exposing animals to the action of x-rays before section. When pieces were exposed to the radiations only a small blastema developed. Histological observations showed an absence of mitosis in all irradiated pieces while controls showed parenchymal cells in division. Because of the differentiation of imperfect eyes in one case following treatment of the entire animal, these authors suggested that x-rays affected growth processes more than differentiation. Later work with x-rays (Curtis, 1928) and radium (Wiegand, 1930) confirmed the work of Bardeen and Baetjer and showed that large doses selectively destroyed parenchymal cells.

Wolff and Dubois (1948) demonstrated cellular migration in regeneration by grafting a piece of normal tissue into the irradiated anterior 2/5 of the *Euplanaria lugubris*. Donor pigment and cells were found to have migrated through the irradiated host tissues and formed a blastema. The duration of migration varied directly with the distance covered to the regenerating surface.

Curtis and Schulze (1934) made direct counts of the parenchymal cells in species capable of regeneration (*Euplanaria agilis, P. maculata*) and in others possessing a limited capacity to regenerate (*Procotyla fluviatilis*). The only cells observed to be in mitosis in this study were the free parenchymal amoebocytes. Their observations indicated that the capacity to reconstitute is correlated with the number of these cells which they suggest are present as a persistent embryonic stock. Hyman (1951), however, holds that this does not explain the capacity of a post-pharyngeal piece of *P. fluviatilis* to regenerate a tail and not a head.

Colchicine has been used to study cellular changes in limb regeneration in larval urodeles. Thornton (1943) treated larvae of *Amblystoma punctatum* and *A. opacum* with colchicine solutions and found that limb stumps of larvae kept in 1 : 1,500 continuously, from the time of amputation, completely failed to regenerate. While the mechanism of colchicine inhibition of regenerating limbs was not investi-

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gated in this work the author suggested that it was no doubt due to the inhibition of mitosis.

Since work on planarian regeneration has shown that cells migrate from the general parenchyma to the site of injury and contribute to the restoration of the lost parts by division, it appeared that colchicine might be useful in the study of planarian reconstitution. Because colchicine effectively inhibits cells in division (and possibly in migration) in other organisms, the present work was undertaken in an attempt to answer, at least in part, some of the following questions concerned with colchicine effects on planarian reconstitution: (1) Does colchicine inhibit regeneration of planarian pieces? (2) If so, how does this affect the head frequency gradient found in normal regeneration? (3) Is the process of fission altered by colchicine? (4) Are the effects of colchicine treatment reversible? (5) What effect, if any, does colchicine have on physiological dominance in reconstituting pieces of planarians? (6) How does colchicine affect differentiation?

Materials and Methods

The material used in this investigation was the flatworm, *Dugesia dorotocephala*. The stocks were taken from spring-fed streams leading into the Fox River at Cary, Ill. and the Des Plaines River at Schiller Park, Ill. During the course of this work large stocks were successfully maintained in the laboratory. Tap water, that had been aerated for at least 24 hours, was used exclusively. Beef liver was fed approximately twice a week. Only animals that had become adjusted to laboratory conditions and were starved for 7–10 days were used in the final experiments. Unless otherwise stated the animals were 14–16 mm. in length in all experiments. Control and experimental animals were always taken from the same stock.

Studies were made on pieces of various lengths. After sectioning in water, groups of similar pieces were placed in aerated tap water (control) and into various test solutions (prepared with tap water) of colchicine. During the fall, winter and spring the animals were allowed to reconstitute at room temperatures (21–23° C.). During the summer the bowls containing the control and experimental pieces were kept on the water table where fluctuations in temperature were not excessive (17–20° C.).

Preliminary tests showed that solutions of $M/1{,}000$ to $M/4{,}000$ colchicine were excessively toxic and that pieces or entire animals exposed to these concentrations cytolyzed within a few days. Similar tests also showed that $M/15{,}000$ was at the lower limit of concentrations yielding colchicine effects. Thus, concentrations ranging from $M/5{,}000$ to $M/15{,}000$ were used in all experiments reported in this work.

Experimental

1. *Effects of colchicine on regeneration in transverse pieces from different body levels*

Pieces, approximately 1/8th the length of 14–16 mm. animals, taken from different body levels were placed directly into $M/5{,}000$ and $M/10{,}000$ colchicine

for periods of 2, 5, 7 and 10 days after which they were returned to water. Daily observations were made until the 10th day. This series included 250 animals (1250 pieces).

Control pieces differed in degree of head regeneration at various levels. Anterior (A) pieces developed almost 100 per cent normal heads while pieces from more posterior levels showed various degrees of head inhibition. At the most

TABLE I

Designation of regulation values

Type	Assigned regulation value	Control
I	100	Old tissue shows regulation to typical form and proportion of new individual. Head types vary according to level of origin of piece.
		Experimental—Colchicine treatment
II	85	Old tissue shows slightly less than normal regulation to new form and proportion; normal head and tail develop.
III	70	Old tissue shows little regulation to new form and proportion; normal or near normal head and tail develop.
IV	55	Old tissue shows no apparent regulation to new form and proportion; small anterior blastema with eyes develops and a posterior blastema may or may not develop.
V	40	Old tissue shows no apparent regulation to new form and proportion; small anterior blastema without eyes develops and a posterior blastema may or may not develop.
VI	25	Old tissue shows no regulation to new form and proportion; no blastema develops but eyes appear in the anterior cut surface.
VII	10	Old tissue shows no regulation to new form and proportion; pieces remain unchanged since time of cut.

posterior levels (E) almost all were headless.[3] Although many of the control pieces showed head inhibition almost all showed regulation in general body form. That is, the regenerating piece decreased in width and extended in length when compared to the original section cut from the parent.

[3] Head inhibition in short transverse pieces has been described many times (Child, 1911, 1912, 1914, 1920; Child and Watanabe, 1935; Watanabe, 1935; Rulon, 1936; Watanabe, 1941). Such inhibition appears to be the result of two factors, namely, the level from which the piece was taken and the intensity of factors originating from the posterior cut surface (Buchanan, 1922).

It was immediately noted that pieces treated with colchicine could not be compared with the controls on the basis of head inhibition since extended treatment, with the higher concentrations, resulted in little or no new tissue. When such pieces were removed to water those surviving regulated (after 2–3 weeks)

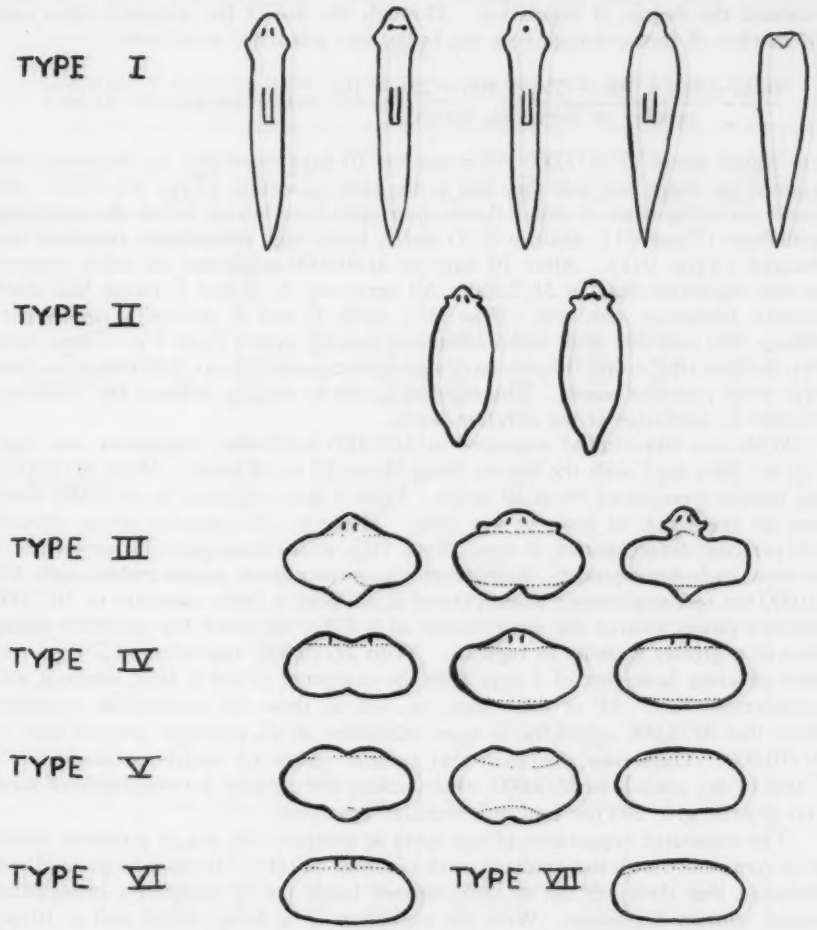

FIGURE 1. Classification of types of regenerates obtained subsequent to colchicine treatment.

to normal body form and regenerated almost 100 per cent typical heads with normal or supernumerary eyes. Since the present experiments were based on observations made 10 days after section, the pieces were classified according to the degree of regulation at that time (Table I, Fig. 1). All regenerates were

classified as to one of 7 types. Type I was the control and was classified as 100 per cent regulated. Types II to VII included those ranging from slightly less than normal regulation to a total lack of change from the time of section. In order to compare data from different experiments (levels, concentrations, etc.) a *Regulation Index* was devised by assigning each type a numerical value which roughly indicated the degree of regulation. Through the use of the assigned value and the number of forms of each type the Index was calculated as follows:

$$\frac{100n + 85n + 70n + 55n + 40n + 25n + 10n}{\text{number of surviving pieces}} = \text{R I (Regulation Index)}$$

One-eighth pieces in $M/5{,}000$ colchicine for 10 days developed no blastemae and retained the shape and size they had at the time of section (Type VI–VII). All pieces surviving from A and B levels developed eyes buried below the overlying epithelium (Type VI), while at C, D and E levels high percentages remained unchanged (Type VII). After 10 days in $M/10{,}000$ colchicine all levels showed greater regulation than in $M/5{,}000$. All surviving A, B and C pieces had small anterior blastemae with eyes (Type IV), while D and E showed a sizable percentage (40 and 36) with small blastemae lacking eyes (Type V). These data then indicate that A and B levels have a greater capacity for eye differentiation than have more posterior pieces. The regulation indices roughly indicate the inhibition effected by colchicine at the different levels.

With two days initial exposure to $M/5{,}000$ colchicine, regulation was high (by the 10th day) with the indices being above 82 at all levels. With $M/10{,}000$, the indices were above 94 at all levels. With 5 days exposure to $M/5{,}000$ there was no regulation of form at any level. However, the anterior pieces showed 100 per cent differentiation of eyes (Type IV), while many posterior pieces failed to show such development. Regulation was greater in all pieces treated with $M/10{,}000$ but best at posterior levels (D and E). With a 7-day exposure to $M/5{,}000$ anterior pieces showed the same results as a 5-day exposure but posterior pieces showed a greater capacity to regulate. With $M/10{,}000$, regulation at 7 days was little different from that of 5 days with the exception of the B level where it was considerably less. All of these data, as well as those for continuous exposure, show that $M/5{,}000$ colchicine is more inhibitory at all exposure periods than is $M/10{,}000$. These data also show that anterior pieces (A and B) exposed for 5, 7 and 10 day periods to $M/5{,}000$, while lacking the capacity for regulation of form and growth, give 100 per cent differentiated eye spots.

The consistent appearance of eye spots at anterior, but not at posterior levels, is in conformity with the gradient work of Child (1911). It must be pointed out, however, that these are not in differentiated heads but in completely unregulated pieces without blastemae. With the exception of a 5-day initial and a 10-day continuous exposure to the high concentrations, posterior pieces showed greater regulation and less susceptibility to the toxic effects of colchicine than did anterior pieces.

In general, these experiments have shown that posterior pieces have greater capacity for blastema formation and viability while anterior pieces have a greater capacity for differentiation of eye spots.

2. A critical period in reconstitution as determined by the use of colchicine

Since the data on transverse pieces, in high concentrations of colchicine, showed that 5 days' exposure was more lethal and permitted less regulation than 7 days (pieces D and E in *M*/5,000 colchicine) a critical period in development, centering around 5 days, was indicated. In order to identify this period more accurately

TABLE II

Comparison of Regulation Indices of 1/6th pieces when treated with M/5,000 and M/7,000 colchicine for 1–9 days after section. (Data in per cent.)

Level	1 day		2 days		3 days		4 days		5 days	
	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index
A	0	100								
B	0	100								
Control C	0	100								
D	0	100								
A	0	91.1	0	97	48	82.6	38	48.5	30	55.6
B	0	94.6	2	78	62	85	72	35.6	62	43.9
M/5,000 C	16	98.9	0	90.4	48	83.2	40	36.5	60	70
D	0	100	0	94.6	18	75.3	11	22.3	22	63.2
A	0	100	0	100	0	100	0	53.2	0	100
B	0	100	0	98.8	44	95.9	0	52.6	32	79.8
M/7,000 C	0	100	0	98.8	20	100	0	40	0	94
D	0	100	0	100	0	91.6	0	55	0	96.8

Level	6 days		7 days		8 days		9 days			
	Dead	Index	Dead	Index	Dead	Index	Dead	Index		
A										
B										
Control C										
D										
A	40	17.5	80	25	80	15	100	10		
B	100	16.5	98	25	100	11.1	100	10		
M/5,000 C	80	25.6	96	13	80	10.6	100	13		
D	46	64.7	96	20	100	13	100	13		
A	64	65	84	12.4	72	14.2	96	25		
B	84	55.5	92	25	92	25	96	10		
M/7,000 C	60	59.5	96	16	92	12.3	100			
D	56	90	60	37.8	92	10	96	25		

(if such actually existed) a further study was made in which slightly larger pieces were exposed to colchicine for more closely graded intervals.

In these experiments, pieces approximately 1/6th the post-cephalic length were used. These pieces were exposed to *M*/5,000 and *M*/7,000 colchicine for 1, 2, 3, . . . 9 days. At the end of each period the pieces were returned to water.

Records were made on the tenth day. An additional analysis was undertaken by treating the pieces with *M*/5,000 colchicine beginning at different time intervals after section. The data, showing treatment with colchicine immediately after cutting, are shown in Table II while those showing treatment at delayed intervals are shown in Table III.

Because so many data are involved (1,075 animals or 4,300 pieces) only Regulation Indices and viability for the different levels are given. It is shown in Table II that exposure for 4 days (not 5, as suggested in the previous section) is more effective in inhibiting growth, and regulation, in both *M*/5,000 and *M*/7,000, than either 3 or 5 days. This difference, relative to exposure time, could not be found in viability (as shown for 1/8th D and E pieces) possibly because the pieces in the present experiment were slightly larger.

The critical period for regeneration and regulation was not shown when pieces were placed into *M*/5,000 colchicine on the 4th day after section (Table III).

TABLE III

Regulation Indices of 1/6th pieces when kept in water for 1–6 days after section and placed into M/5,000 colchicine for the remainder of a 10-day reconstitutional period. (Data in per cent.)

Level	1 day		2 days		3 days		4 days		5 days		6 days	
	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index
A	0	100										
B	0	100										
Control C	0	100										
D	0	100										
A	2	54.6	2	54.3	32	54.5	40	56.3	28	62.5	42	64.3
B	26	52.5	42	55	44	53.3	56	60.5	42	64.6	38	55
M/5,000 C	8	48.7	26	47.2	48	48	56	55.7	22	51.1	22	68
D	0	49.3	0	57.2	30	51.5	34	56.3	14	74.8	30	70.4

However, these data show that 4-day regenerates from all levels are more sensitive to the lethal effects of colchicine than are pieces which have regenerated for 3 or 5 days.

Sensitivity of developmental processes to inhibiting agents has been shown to be associated with cleavage and differentiation. Rulon (1950) demonstrated that the fertilized eggs of *Dendraster* were more sensitive to thiourea treatment than were blastulae. Blastulae tolerated high concentrations that were lethal to newly fertilized eggs. Changes associated with reconstitution appear to include an initial adjustment to the effect of section and perhaps dedifferentiation, followed by reorganization including cell proliferation with the final return to species character. It is suggested that the critical period noted here, with its counterpart in normal development, is associated with that time in development when reorganization and cell proliferation are occurring in the reconstitutional process. Histological studies, to be reported later, should verify this proposal.

3. The effect of colchicine on regeneration when whole animlas are treated prior to section

The preceding work showed that planarian pieces were inhibited in both reconstitution and regulation of form when exposed to certain concentrations of colchicine. Obviously the colchicine was exerting its effects on tissue which had been stimulated to regenerate by cutting. The problem which then presented itself dealt with the effect of colchicine on unactivated tissue. To test this, uninjured entire animals were exposed to various concentrations of the agent for 2–6 days after which they were sectioned into 1/6th pieces and permitted to reconstitute in water. Because these experiments were performed in summer, all intact animals and pieces were kept on the water table at 17–19° C. The data were taken on the tenth day following section. This experiment included 200 animals. In general, pieces from treated animals behave much the same way as if they, themselves, had been exposed to the agent. That is, there was prolonged inhibition of regulation and blastema formation in the higher concentrations. The most

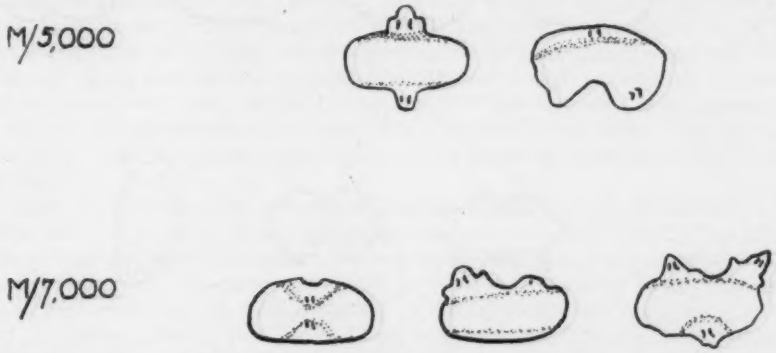

FIGURE 2. Types of heteromorphic forms obtained from 1/6th pieces of 14–16 mm. animals pre-treated with colchicine different lengths of time (*M*/5,000 for 4 days; *M*/7,000 for 6 days). Whole animals and pieces maintained at 17–19° C.

important difference between the regenerates obtained here and those from treatment following section occurred in the development of a high percentage (36 per cent at D level) of heteromorphic forms when the whole animals were treated for 4 days with *M*/5,000 and 6 days with *M*/7,000 colchicine. Regenerates classified as heteromorphic included: (1) simple bipolars, (2) unregulated bipolars (with or without blastemae), (3) poorly regulated forms with two heads at the anterior end, and (4) poorly regulated forms which have regenerated two heads anteriorly and one posteriorly (Fig. 2).

A slightly different series was prepared in which the intact animals were kept at room temperature (21–23° C.) in *M*/2,000, *M*/5,000 and *M*/10,000 colchicine for only two days. After this exposure the animals were cut into 1/8th instead of 1/6th and permitted to reconstitute in water, also at room temperature. This experiment included 225 animals.

This second series showed that increased temperature, with a reduction in

exposure time and size of piece, resulted in considerably less regulation when whole animals were treated with $M/5{,}000$ prior to section. When treated with $M/10{,}000$, under these changed conditions, regulation was also decreased, but it was noted that approximately 50 per cent of those pieces classified as Type V (small blastemae, no eyes) had two separate blastemae at each end of the piece.

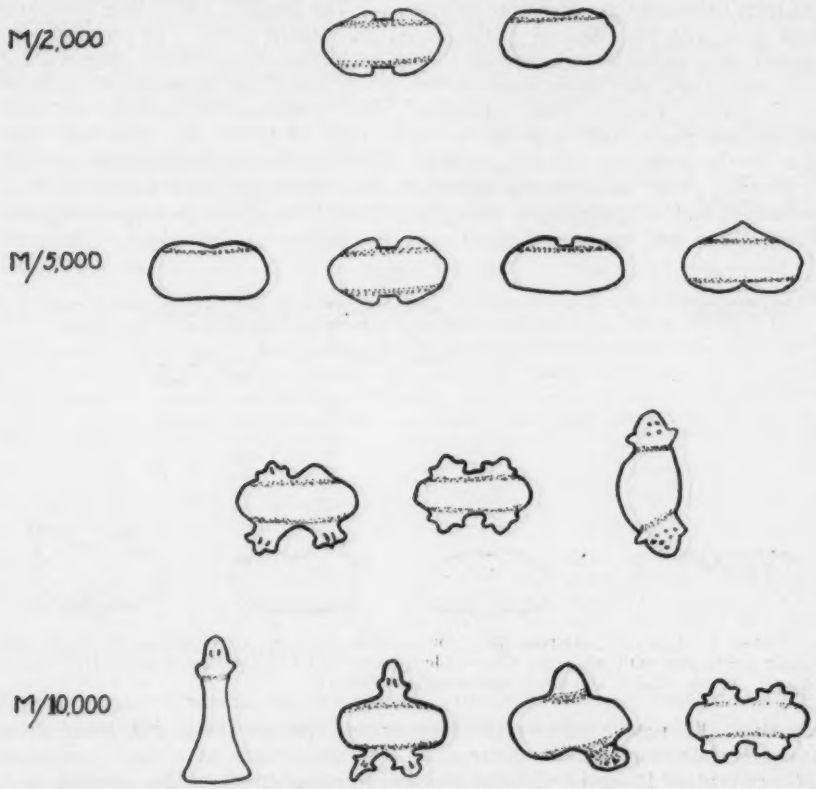

FIGURE 3. Types of regenerates obtained from 1/8th pieces of 14–16 mm. animals pre-treated with colchicine for two days. Whole animals and pieces maintained at 21–23° C.

High percentages (28–44) of heteromorphic forms also appeared. In general, these were similar to those found in the first series but with the addition of a 5th type which had regenerated two heads at each end of the piece (Fig. 3). At D level more than 50 per cent of these forms had 3 or 4 heads.

DISCUSSION AND CONCLUSIONS

This work has shown that certain concentrations of colchicine markedly inhibit regeneration and regulation to normal body form in planarians. It has shown that the degree of inhibition was roughly proportional to the concentration

and that the character of the regenerate showed a high degree of consistency. In all experiments using solutions below the toxic limits it was observed that pieces which survived early treatment with colchicine developed normal heads and attained normal body form and proportion by 10–15 days after return to water. The head frequency gradient (Child, 1913, 1914; Buchanan, 1922; Rustia, 1925; Rulon, 1936, 1938; Watanabe, 1935, 1941) was, therefore, obliterated by this agent. In tap water, short transverse pieces from D and E levels regenerate largely into headless, or near headless forms. It has been found (Buchanan, 1922; Rulon, 1948 and others) that factors inhibiting normal head regeneration arise at the site of posterior section and that these factors may be blocked by nerve anaesthetics and other agents. Such head-inhibiting factors are of short duration since a 24-hour delay in making a posterior cut will permit the development of 100 per cent normal heads (Child and Watanabe, 1935). The present experiments indicate that since colchicine prevents regenerative changes in the piece over an extended period, the head-inhibiting posterior effects have subsided by the time the pieces are returned to water.

The gradient of eye-spot formation, however, was still apparent after 10 days since exposure to high concentrations for this period of time resulted in 100 per cent eye-spots in pieces from anterior levels while no more than 56 per cent of the posterior pieces showed such development. Even though growth and regulation of these pieces was totally inhibited, differentiation was expressed in the appearance of eyes in pieces otherwise unchanged since the time of isolation. This indicates that while colchicine inhibits regeneration it does not at the same time totally inhibit differentiation.

Many investigations have shown a correlation between the presence of nerve tissue and the capacity to reconstitute to normal. Olmsted (1922) has shown that certain polyclads can restore missing parts only if cephalic ganglia are left intact. Silber and Hamburger (1939) and Beyer and Child (1930) observed head development on the medial surface of lateral pieces and suggested that the nervous system plays a role in the localization of head regeneration through its influence on the condition of the cells near it. In the present experiments it is possible that the appearance of eyes below the epithelium at the anterior cut surface of a piece is also correlated with the localizing influence of the nerve cords.

The occurrence of regenerates possessing 3–4 blastemae or 3–4 heads may find its explanation in the obliteration of the medio-lateral differential or gradient. All head formation in normal reconstitution is initiated at the medial point of a cut surface and progresses laterally. The occurrence of two blastemae or two heads at a cut surface (Figs. 2, 3) indicates that the medio-lateral gradient is reduced. A high percentage of pieces taken from animals treated with colchicine before section showed the formation of paired blastemae or heads at both cut surfaces. Apparently colchicine effectively destroys the medio-lateral differential and in so doing frees each side of the piece for independent differentiation. The localizing influence of the nerve cords may be operative here also, as it appears to be in eye spot differentiation after treatment with colchicine.

Summary

1. Colchicine was shown to inhibit regeneration in pieces of *Dugesia dorotocephala*. In addition it was shown to inhibit regulation to normal form.

2. Within certain limits of concentration, the inhibitory effects of colchicine were reversible. Two to three weeks after return to water pieces from all levels developed normal heads (some with supernumerary eyes). Thus, through the use of colchicine it was possible to obliterate the head frequency gradient characteristic of this species.

3. While colchicine was found to exert a marked inhibition of blastema formation in the reconstitution of planarian pieces it did not have the same effect on eye-spot differentiation. Pieces treated with higher concentrations of the agent failed to show any new tissue at the anterior and posterior cut surfaces by 10 days after section. Eye-spots, however, differentiated beneath the covering epithelium in anterior pieces.

4. Heteromorphic forms with 3–4 heads developed from pieces taken from whole animals exposed to colchicine. This indicates that the medio-lateral differential was reduced subsequent to colchicine treatment and that the two lateral halves of the piece underwent independent differentiation.

5. With the use of colchicine evidence was obtained that the 4th day of reconstitution represents a critical period in development. That is, pieces treated with $M/5{,}000$ colchicine for 4 days, and then returned to water, showed more inhibition than pieces treated for 3 or 5 days.

LITERATURE CITED

Bardeen, C. R., and F. H. Baetjer, 1904. The inhibitive action of roentgen rays on regeneration in planarians. *J. Exp. Zool.*, **1**: 191–195.

Beyer, K. M., and C. M. Child, 1930. The reconstitution of lateral pieces of *Planaria dorotocephala* and *Planaria maculata*. *Physiol. Zool.*, **3**: 342–365.

Buchanan, J. W., 1922. The control of head formation in planaria by means of anaesthetics. *J. Exp. Zool.*, **36**: 1–48.

Child, C. M., 1911. Experimental control of morphogenesis in Planaria. *Biol. Bull.*, **20**: 309–331.

Child, C. M., 1912. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. IV. Certain dynamic factors in the regulatory morphogenesis of *Planaria dorotocephala* in relation to the axial gradient. *J. Exp. Zool.*, **13**: 103–152.

Child, C. M., 1913. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. V. The relation between resistance to depressing agents and rate of metabolism in *Planaria dorotocephala* and its value as a method of investigation. *J. Exp. Zool.*, **14**: 153–206.

Child, C. M., 1914. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. VII. The stimulation of pieces by section in *Planaria dorotocephala*. *J. Exp. Zool.*, **16**: 413–443.

Child, C. M., 1920. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. X. Head frequency in *Planaria dorotocephala* in relation to age, nutrition and motor activity. *J. Exp. Zool.*, **30**: 403–418.

Child, C. M., and Y. Watanabe, 1935. The head frequency gradient in *Euplanaria dorotocephala*. *Physiol. Zool.*, **8**: 1–40.

Curtis, W. C., 1928. Old problems and a new technique. *Science*, **67**: 141–149.

Curtis, W. C., and L. M. Schulze, 1934. Studies upon regeneration, I. The contrasting powers of regeneration in Planaria and Procotyla. *J. Morph.*, **55**: 477–513.

Hyman, L. H., 1951. The invertebrates: Platyhelminthes and Rhynchocoela. Vol. 2, p. 182–190. McGraw Hill Book Co., New York.

Olmsted, J. M. D., 1922. The role of the nervous system in the regeneration of polyclad Turbellaria. *J. Exp. Zool.*, **36**: 49–56.

Rulon, O., 1936. The effects of carbon dioxide, the hydrogen ion, calcium and experimental conditioning on reconstitution in *Euplanaria dorotocephala*. *Physiol. Zool.*, **9**: 170–203.

RULON, O., 1938. Single and combined effects of cyanide and methylene blue on reconstitution in *Euplanaria dorotocephala*. *Physiol. Zool.*, **11**: 203–213.

RULON, O., 1948. The control of reconstitutional development in planarians with sodium thiocyanate and lithium chloride. *Physiol. Zool.*, **21**: 231–237.

RULON, O., 1950. The modification of developmental patterns in the sand dollar by thiourea. *Physiol. Zool.*, **23**: 248–257.

RUSTIA, C. P., 1925. The control of biaxial development in the reconstitution of pieces of planaria. *J. Exp. Zool.*, **42**: 111–142.

SILBER, R. H., AND V. HAMBURGER, 1939. The production of Duplicitas cruciata and multiple heads by regeneration in *Euplanaria tigrina*. *Physiol. Zool.*, **12**: 285–301.

THORNTON, C. S., 1943. The effect of colchicine on limb regeneration in larval Amblystoma. *J. Exp. Zool.*, **92**: 281–291.

WATANABE, Y., 1935. Head frequency in *Euplanaria maculata* in relation to the nervous system. *Physiol. Zool.*, **8**: 374–394.

WATANABE, Y., 1941. Effects of modified Ringer's solution on head regeneration in *Dugesia dorotocephala*. *Physiol. Zool.*, **14**: 316–327.

WIEGAND, K., 1930. Regeneration bei Planarien und Clavelina unter dem Einfluss von Radiumstrahlen. *Zeitschr. Wiss. Zoll.*, **136**: 255–318.

WOLFF, E., AND F. DUBOIS, 1948. Sur la migration les cellules de regeneration chez les Planaires. *Rev. Suisse Zool.*, **55**: 218–227.

CHEMORECEPTION IN INSECTS AND THE ACTION OF DDT[1]

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The unstabilizing action of DDT on many excitable tissues has been firmly established by work in several laboratories (see Roeder and Weiant, 1948, 1951 for references). However, as Roeder and Weiant demonstrated, not all irritable tissues are equally sensitive. At high concentrations of DDT they observed multiple discharges (impulse trains) in cockroach motor nerves, accounting for the tremors observed in isolated legs. At much lower concentrations, motor nerves were unaffected, but certain proprioceptors were selectively unstabilized, also firing short trains or sequences of impulses in place of single spikes. These barrages of sensory input were taken to account for the hyperexcitability and chaotic behavior of insects in the early stages of DDT poisoning.

If the selective action of DDT extends also to different types of receptor, DDT might be a useful tool for the separation and study of the sense modalities mediated by the many varieties of sensilla scattered over the cuticle of insects. Furthermore, since there are strains of housefly highly resistant to DDT, a comparison in different strains of a sense modality affected by DDT might provide information about the sense concerned and the mechanism of resistance to DDT.

With this in mind the action of DDT on chemoreceptors is considered in this paper. An attempt is made to correlate information on receptor function which has been obtained by electrophysiological methods with that derived from behavioral studies.

1. Electrophysiological studies relating chemical stimulation and DDT action

In the course of experiments on chemoreception (Roys, 1954) the vapors of volatile substances such as benzene and toluene were applied in increasing measured concentrations to intact roaches (*Periplaneta americana* L.) and to isolated legs and antennae. It was found that a concentration range in which the free insect appeared to show mild awareness of the chemical also caused a steady increase in the number of afferent nerve impulses recorded from electrodes in the isolated appendage. At higher concentrations, which the animal tried to avoid, electrical activity in the nerves of detached legs changed from a steady high level to a fluctuating pattern of great bursts alternating with periods of relative inactivity. This, and the fact that DDT is capable of producing abnormal impulse trains in certain proprioceptors of the insect leg, suggested an experiment in which the actions of benzene or toluene and DDT were combined.

[1] The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army, and Tufts College. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

Isolated roach or fly legs or segments of legs were pierced by hot-drawn tungsten electrodes. Afferent electrical activity in the leg was amplified and examined with the aid of an oscillograph and loud speaker. Each experiment was also recorded on magnetic recording tape for later study.

To minimize mechanical disturbance of the preparations the chemicals were applied as vapors. DDT was volatilized by heating the crystals in a glass tube and gently blowing the vapor over the preparation, taking care not to overheat the leg. Typical DDT trains recurred quite regularly and were unaffected by bending large spines. However, toluene vapor from an olfactometer had a well defined effect on the DDT trains, increasing their duration and frequency.

The effect is illustrated in Figure 1. The metathoracic femur of a housefly (*Musca domestica* L.) was mounted with an electrode in each cut end and covered with vaseline. The vaseline blanket limited the number of receptors responding to external stimulation, and provided a relatively simple pattern of afferent discharge. In the untreated preparation (1A) a single fiber was firing somewhat irregularly. Exposure to the vapor of toluene (1B) brought in several other fibers and increased the frequency of discharge, which had not quite returned to the resting level after the toluene had been removed (1C). Three minutes after a brief exposure to DDT vapor (1D) a sequence of well-defined DDT trains appeared, taking the place of the sequence of single spikes. However, the number of exposed receptors had been so reduced by the vaseline coating that the DDT trains were relatively infrequent in the absence of toluene. Exposure of the DDT-treated preparation to toluene (1E) was found to influence the repetition frequency of whole trains in a manner similar to the way in which it affected the single action potentials before DDT treatment. Also, several previously inactive fibers, also firing in trains, were brought by toluene to the discharge level so that the total number of impulses in unit time for a given toluene concentration was much greater after treatment with DDT. Removal of the benzene (1F) led to a return to a condition in which, once more, only occasional trains appeared. The effects of toluene can be obtained and removed many times on the same preparation, but the effects of DDT are irreversible under these conditions. Similar results were obtained from the tarsi of roaches.

This gives weight to the suggestion (Roeder and Weiant, 1948) that DDT in itself does not stimulate receptors, but changes their pattern of response to normal stimuli from discharge of single spikes to repeated high frequency trains of impulses. As with proprioception, one might expect DDT poisoning to produce excessive reflex behavior due to the increased numbers of impulses delivered by each chemoreceptor at a given level of stimulation. Behavioral confirmation of this is described in the next section.

2. DDT and contact chemoreceptor thresholds

When the tarsi of various insects come in contact with certain "acceptable" compounds, the insects respond by extending the proboscis and attempting to feed. This reflex has been the basis for numerous studies of tarsal chemoreception, notably by Minnich, Frings, and Dethier (see Dethier and Chadwick, 1948, for references). Modifications from their techniques were used in the studies described here.

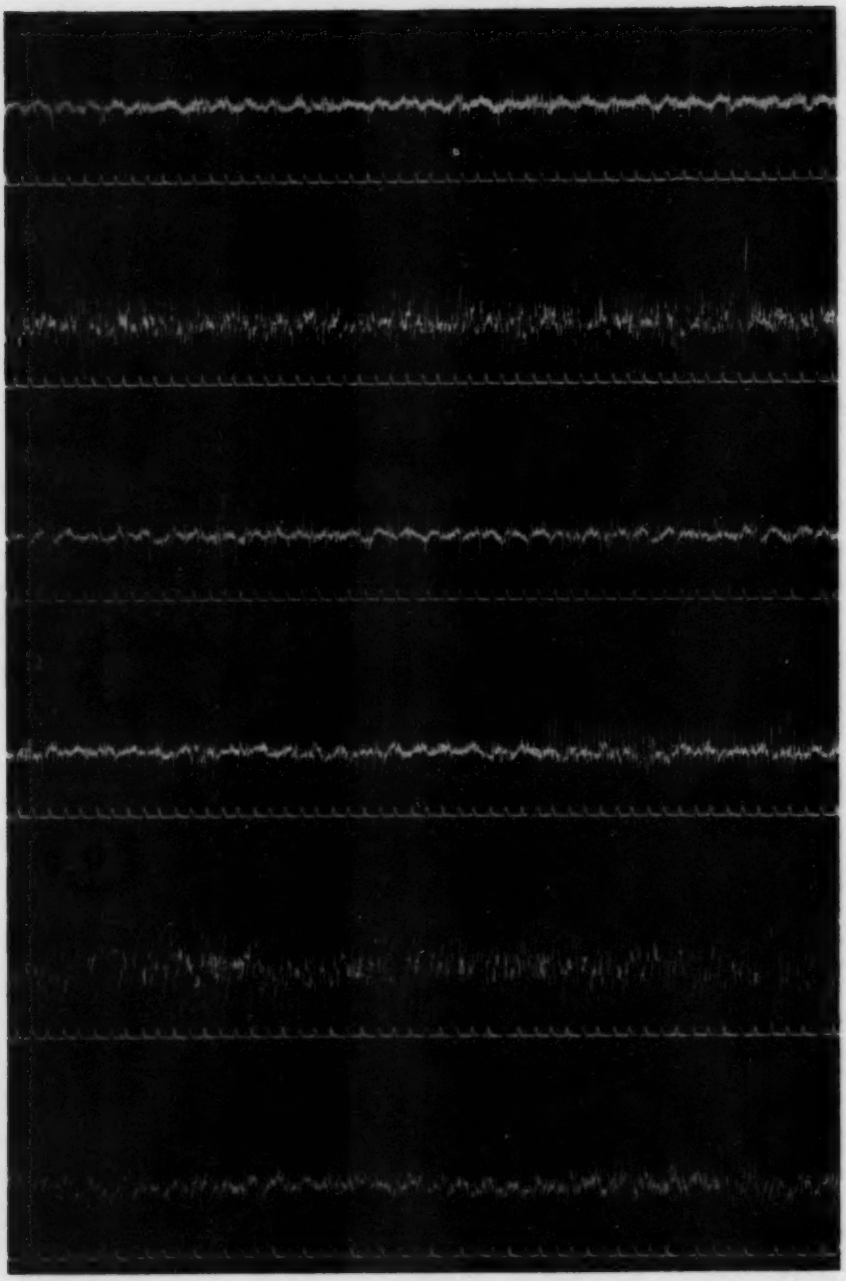

FIGURE 1.

Tarsal thresholds were determined for two strains of houseflies (*Musca domestica* L.), one DDT-sensitive, the other DDT-resistant, obtained from Dr. L. E. Chadwick of the Army Chemical Center, and for DDT-sensitive blowflies (*Phormia regina* Meigen) obtained from Professor V. G. Dethier of the Johns Hopkins University.

The larvae were reared on a milk-yeast-agar medium at 30° C. Pupae and adults were kept at room temperature (21–25° C.). Emerging flies were removed daily and given access to a supply of 0.1 *M* sucrose. One day later flies to be used were anesthetized with carbon dioxide and attached by their wings to paraffined sticks. When volatile substances were to be tested, olfactory receptors (antennae, palpi and labellum) were extirpated at this time.

Thresholds were determined the following day by one of several procedures. For sucrose acceptance thresholds the flies were first allowed to drink distilled water to repletion. Each fly was then held for two seconds with its tarsi in one of a series of sucrose concentrations. Proboscis extension within this time constituted a positive response. If the fly failed to respond, it was tested on 0.1 *M* sucrose, a concentration to which all normal flies should respond. Response here was taken to indicate that the fly was normal in so far as its feeding response was concerned, and it was scored as negative to the previously tested solution. Flies not responding to 0.1 *M* sucrose were considered to be injured or grossly abnormal and data on them were discarded.

Rejection thresholds for NaCl and ethanol were determined in two ways. The various concentrations of the compound to be tested could be made up in 0.1 *M* sucrose, in which case the rejection threshold was the salt or alcohol concentration just preventing the normal positive response to sugar. Or, having denied the flies water before testing, the solutions could be made up with distilled water (no sucrose), the rejection threshold obtained being relative to the thirstiness of the flies. Thresholds against water were much more difficult to obtain than thresholds against sugar, but a few were determined to show whether the action of DDT on sugar receptors masked an effect of DDT on "rejection" chemoreceptors. In either case the flies were given ten seconds to reject the test solution. Extension through the ten second period constituted a positive response (sub-threshold salt or alcohol). Flies failing to respond, or retracting within ten seconds, were checked for two seconds on 0.1 *M* sucrose or distilled water, depending on the type of experiment. Flies responding here were classed as negative; those not responding at all were discarded. In no case was a fly allowed to drink a test solution.

The usual method of DDT application was to place the tarsi of the mounted flies in contact with a DDT-coated glass plate for five minutes immediately before testing. Sensitive houseflies developed symptoms of poisoning seven to eight minutes after exposure at the concentration used—0.01 milligram DDT per square

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FIGURE 1. The effect of toluene vapor on electrical activity in afferent fibers of the housefly femur before and after treatment with DDT. A. Untreated preparation. B. Increased activity upon exposure to toluene vapor. C. Toluene vapor withdrawn. D. Three minutes after exposure to DDT vapor. E. Effect of toluene vapor on the DDT-treated preparation. F. Toluene vapor withdrawn, leaving only DDT trains. Time marks, 10 millisecond intervals. For further explanation see text.

centimeter (approx. 9.3 mg./sq. ft.) deposited from acetone solution. To determine whether the cuticular surface of the sensitive tarsal chemoreceptor was the site of selective action by DDT, the insecticide was also applied to another area. In these experiments the meso- and metathoracic legs were immobilized in paraffin ("two-leg flies") and a small droplet of DDT in mineral oil (one milligram per milliliter) was applied to the tip of the abdomen. To answer the question of whether cuticular impermeability barred DDT from the receptors of resistant flies sucrose thresholds were determined for flies injected with DDT. Sensitive as well as resistant flies were run as a check on the method. One per cent DDT in tri-ethylene glycol was serially diluted with Pringle's insect Ringer to 10^{-5} for

TABLE I

Tarsal sucrose acceptance thresholds of Musca and Phormia with and without various DDT treatments

Species	DDT sensitivity	Treatment	Median molar threshold	Log median threshold	Standard error log threshold	Regression response on concentration	Standard error regression	Total flies tested
a. Musca	sensitive	control	.0067	−2.176	±.039	2.00	±.16	479
		tarsal DDT	.00074	−3.129	.029	1.37	.24	201
b. Musca	resistant	control	.0043	−2.365	.068	1.59	.21	204
		tarsal DDT	.0041	−2.389	.063	1.79	.25	204
c. Phormia	sensitive	control	.0020	−2.689	.053	2.16	.28	192
		tarsal DDT	.00043	−3.363	.083	1.50	.22	162
d. Phormia (2-leg)	sensitive	control	.013	−1.887	.046	3.80	.77	66
		tarsal DDT	.0026	−2.585	.055	3.29	.77	64
		abdominal DDT	.0029	−2.536	.059	2.90	.72	63
e. Musca	sensitive	tarsal DDT	.0047	−2.331	.075	1.24	.19	216
		injected DDT	.012	−1.913	.053	2.34	.32	216
f. Musca	resistant	tarsal DDT	.0054	−2.271	.055	2.35	.30	132
		injected DDT	.0064	−2.191	.058	2.98	.37	132

resistant flies and 10^{-6} for sensitive flies; 0.004 milliliter of this was injected into the thorax, giving doses of 0.04 and 0.004 microgram per fly, respectively.

Whenever there were sufficient data, a probit analysis of the results was made (Finney, 1952). In other cases median thresholds were estimated by graphic interpolation.

The tarsal chemical thresholds obtained are listed in Tables I and II. DDT lowered the tarsal sucrose threshold of DDT-sensitive houseflies by a factor of nine, but did not affect the response threshold of resistant flies (I,a,b). The sucrose threshold of blowflies was also strikingly lower following exposure to DDT (I,c).

The method of application was unimportant; DDT picked up by the tarsi, applied in mineral oil to the abdomen (I,d) or injected in saline suspension (I,e) all increased the sensitivity to sucrose. The sucrose thresholds (I,e) of sensitive houseflies following tarsal and internal application of DDT differ by a factor of

three, and at first sight suggest that injected DDT was only half as effective as externally applied DDT in lowering the sucrose threshold. However, this difference can be accounted for by the fact that thresholds of the injected flies varied over a wide range. Only about half of the injected flies appeared to be effectively treated, their thresholds and variance being similar to those of the tarsally poisoned flies in this group. The remaining flies had thresholds scattered about a concentration some nine times higher, but with a similar variance. Possibly the large amount of solution injected interfered with circulation, preventing the transport of DDT to the receptors. The thresholds of flies in I,e and I,f cannot be

TABLE II

Tarsal salt and alcohol rejection thresholds of Musca and Phormia tested against water or sucrose solutions, with and without tarsal application of DDT

Species	DDT sensitivity	Treatment	Median molar threshold	Log median threshold	Standard error log threshold	Total flies tested
NaCl against 0.1 M sucrose						
a. Musca	sensitive	control	1.7	0.22		125
		tarsal DDT	1.7	0.23		70
b. Phormia	sensitive	control	1.4	0.161	±.019	110
		tarsal DDT	1.3	0.130	.018	110
NaCl against water						
c. Musca	sensitive	control	1.0	0.00		30
		tarsal DDT	1.2	0.12		51
Ethanol against 0.1 M sucrose						
d. Musca	sensitive	control	6.8	0.83		59
		tarsal DDT	4.4	0.65		34
e. Musca	resistant	control	3.5	0.55		20
		tarsal DDT	2.5	0.40		21
f. Phormia	sensitive	control	1.7	0.22		44
		tarsal DDT	1.8	0.25		44
Ethanol against water						
g. Musca	sensitive	control	1.1	0.05		15
		tarsal DDT	1.1	0.05		13

compared with those in I,a and I,b, it being a common experience in such tests that thresholds vary widely in different experimental groups.

The threshold of resistant flies was unchanged (I,f), even when DDT was injected in amounts sufficient to poison some individuals. In no case did DDT treatment significantly affect a salt or alcohol threshold, either tested against sucrose or against water (Table II).

It should be pointed out that absolute threshold values changed from time to time over a considerable range, even though an attempt was made to maintain standard rearing and test conditions. The comparisons indicated in Tables I and II represent data collected in concurrent experiments where alternate flies in the

same population were given the various treatments. Some of the data represent lumping of results from duplicate experiments run within a period of a few days. In all these cases the individual experiments were consistent with each other, although slight displacements of the absolute thresholds increase the standard errors. Considerable other data not suitable for inclusion in the tables substantiate the results reported.

In spite of an intensive search in several laboratories there is no electrophysiological evidence of afferent nerve impulses from chemoreceptors concerned with detection of sugar or other substances producing proboscis extension by flies. Nevertheless, perception of such stimuli can be understood only in terms of increased or decreased receptor activity. Because DDT in general increases the activity of irritable units if it affects them at all, and because it also increases the fly's sensitivity to sugar, the simplest explanation of these observations is that there must be chemoreceptors which respond to acceptable compounds by sending nerve impulses more frequently to the central nervous system. DDT would then act by converting each single impulse to a group or train of impulses (Fig. 1), increasing the message rate relative to the supply of information. Chances for central summation, either spatial or temporal, would be increased, and so therefore the likelihood of response. In other words, assuming a steady central excitatory state, if proboscis extension be elicited by a sensory input consisting of a certain number of impulses in a given time interval from a specific group of receptors, then the tendency to high frequency repetition caused by DDT should make possible the requisite input from a smaller number of receptors, and hence from a lower concentration of stimulant.

A somewhat more complicated but also plausible explanation is that the chemoreceptors detecting acceptable compounds are relatively DDT-insensitive. The sensitizing action of DDT would then have to be on some other receptor system, activity in which would facilitate the proboscis response. This idea will be referred to again.

Although salt and alcohol are representatives of different human taste modalities and may also be different to the insect, both are rejected and they can be discussed together here. In no instance was a rejection threshold affected by DDT treatment. Yet, the preceding electrophysiological observations indicate that DDT increases the afferent response to chemical stimulation. Moreover, as demonstrated by the rejection thresholds against sugar compared with those against water, rejection thresholds depend on the intensity of the opposing acceptable stimuli, high sugar concentrations raising the rejection thresholds. And, sugar is a more effective stimulus following DDT treatment. Therefore, DDT must increase the activity of "rejection" receptors to an extent that balances its effect on sugar receptors or their adjuncts. Furthermore, acceptable and unacceptable compounds must act primarily on different receptor units and not in opposite manner on one receptor type. Otherwise one would have to attribute to DDT simultaneous stabilizing and unstabilizing actions on the same cell in order that the rejection threshold remain unchanged. This confirms Dethier's (1953) conclusion that there must be at least two varieties of chemoreceptors on fly tarsi.

DDT applied to the surface of the abdomen or injected into DDT-sensitive houseflies and blowflies lowered sucrose thresholds just as it did when applied to the tarsi. Because neither externally applied nor injected DDT affected the

thresholds of DDT-resistant flies, it seems reasonable to say that cuticular impermeability is not solely responsible for the resistance of this strain. Rather, resistance must include some intrinsic mechanism by which the receptors are protected from the unstabilizing action of DDT. Pratt and Babers (1953) reached a similar conclusion for the thoracic ganglion after studying its DDT-sensitivity in susceptible and resistant houseflies. Although they believe that enzymatic detoxification of DDT is not rapid enough to account for the difference observed between the two strains, it seems possible that a strategic deployment of the protective enzymes to the primary sites of DDT action could account for such resistance.

3. Surface texture and the proboscis extension reflex

In the course of tarsal chemoreception tests on *Phormia* and *Musca* it was observed that an occasional fly would attempt to feed when placed on a clean, smooth surface, especially during the earliest stages of DDT poisoning. Using surfaces at hand it was found that proboscis extension occurred when the tarsi were placed on smooth glass, Lucite, celluloid, polished metals and glazed kymograph paper, but not on newsprint, rough wood, frosted glass or the investigator's finger or clothing. Proboscis extension being the customary criterion of effective stimulation of the "acceptance" receptors, this behavior seemed worthy of further investigation.

Additional observations were made on 338 houseflies mounted on sticks in the manner previously described. After 24 to 40 hours of starvation (until a good response to water was obtained), they were tested in the following manner. Each fly was held so that its tarsi were in contact with either the smooth or rough side of a piece of frosted glass for five seconds, then transferred to the other surface. This was repeated, and if no response was evoked the fly was tested on distilled water. When a positive response was obtained with a dry surface, the fly was repeatedly tested for five-second intervals on the two surfaces.

Of 177 flies responding positively to distilled water, one invariably responded to both surfaces, 155 to neither, and 21 discriminated between the two, responding only to the smooth side. None responded preferentially to the rough. A few *Phormia* were later observed to respond to a clean, smooth surface even though satiated with distilled water. Feeding dry powdered sugar one hour before testing did not alter the proportion of flies responding to surface texture. DDT applied in the manner described above increased the level of response to almost all stimuli.

Like most other behavior patterns the proboscis reflex is not solely the product of a single chemical releasing stimulus, but depends on the balance between facilitating and inhibitory influences from a number of sources, *e.g.*, other chemical stimuli, nutritional state, water balance and mechanical excitation. If these secondary stimuli were to produce a sufficiently favorable state of central excitation, it is possible that spontaneous activity from the chemoreceptors could exceed the threshold for proboscis extension. A background of summating activity from many mechanoreceptors stimulated in unison as by a smooth surface might well contribute to a favorable central state. Rough surfaces should stimulate in a more erratic fashion.

DDT could increase the likelihood of feeding response either by amplifying the activity of the chemoreceptors, or by increasing the number of mechanorecep-

tors active at any one time. Although DDT eventually increases the irritability of certain mechanoreceptors and chemoreceptors, the magnitude of its effect on the sugar threshold is probably indicative of an early action on sugar receptors.

One wonders why flies should be more prone to feed on a smooth surface. Perhaps the vacuum-cleaner type mouth part functions more efficiently here. Also, the resemblance between smooth surfaces and a water surface may be great to a fly.

4. Perception of DDT by houseflies

The fact having been established that DDT can increase the sensitivity of various receptor systems to their appropriate stimuli, there remained an interesting question as to whether the insect can detect DDT itself. Ability to do so would make possible a behavioral type of resistance through selection of strains avoiding the chemical. A rather surprising answer to this question was obtained by

TABLE III

Distribution of houseflies in petri dishes between chemically impregnated and control (acetone-treated) papers

Compound tested	DDT sensitivity	Flies on treated paper	Flies on control paper	Chi-square	Probability	Total flies on paper	Total possible flies on paper
DDT	sensitive	1336	1136	16.2	.01	2,472	12,100
DDT	resistant	792	808	0.16	.5–.7	1,600	9,040
DDT	sensitive	611	506	9.86	.01	1,117	4,800
DDE	sensitive	558	547	0.110	.7–.8	1,105	4,800
1,1-bis(p-chloro-phenyl) ethane	sensitive	564	575	0.106	.7–.8	1,139	4,800

confining flies in chambers with two pieces of paper, one containing DDT, and recording periodically the distribution of the insects.

The procedure was as follows. Seven-centimeter filter papers were prepared with 0.01 milligram DDT or other test compound per square centimeter deposited from acetone solution. After complete evaporation of the solvent the papers were cut in half and paired with acetone-treated control half circles in the bottoms of clean petri dishes. To keep them in place, the papers were dampened with distilled water. Ten flies were placed in each arena and counts of the number of flies on each of the two test half-circles in each petri dish were made every minute from the eleventh through the twentieth minute after the beginning of the experiment. Clean dishes and fresh papers were used for each test.

Under these conditions sensitive flies exposed to DDT in the course of the experiment did not begin to show overt signs of DDT poisoning until thirty minutes or more after introduction into the dishes. Each dish contained one test and one acetone control paper. Both papers and dishes were arranged according to a Latin square design in order to minimize the influence of external factors such as light and temperature gradients, and bias on the part of the observer.

The results are shown in Table III. In both series of tests the data clearly show that DDT-sensitive flies have a predilection for the DDT-treated surface. DDT-resistant flies show no such preference. However, DDT-sensitive flies fail to discriminate between surfaces treated with DDE (1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene) or 1,1-bis (p-chlorophenyl) ethane and acetone controls. This suggests that the factor in the DDT-treated paper which is the basis for preference is connected with the specific physiological activity of DDT rather than with general physical or chemical properties which it presumably shares with the two relatively non-toxic DDT analogs tested.

Proboscis extension and withdrawal are not the only responses to chemical stimulation of the tarsi. When one of the tarsi touches an acceptable substance the fly turns toward that point and begins feeding. Avoidance of noxious chemicals also occurs. Thus stimulation of contact chemoreceptors initiates orientation behavior related to feeding as well as the feeding reflex itself. Probably, the same receptors control both activities. Since it is apparent that there are different groups of receptors responding to acceptable and unacceptable compounds, the preference of DDT-sensitive flies for DDT could be taken to show that DDT selectively potentiates (by causing impulse trains) the sensory input from "acceptance" receptors during the early stages of poisoning, "rejection" receptors being either less sensitive or slower to respond. On this basis a DDT-treated surface could have an illusory attractiveness to flies when first walking on it. This would account for the increased time spent on such surfaces in the experiment. DDT-sensitive flies, by choosing to spend additional time in DDT-treated areas in a partially treated environment contribute to their own destruction. Through loss of ability to detect the insecticide, relatively resistant flies will come in contact with less DDT and further improve their chances of survival.

General Discussion

Among the many receptors on the tarsi of flies several types responding to different modalities of stimulation control the feeding response. These include at least two types of contact chemoreceptor (see also Dethier, 1953), activated by acceptable and unacceptable compounds, releasing or inhibiting the feeding reflex and orientation to food. Under conditions of near-starvation a suitable pattern of tactile stimulation can release feeding behavior. Therefore, one may assume that mechanoreceptors modulate the effects of afferent impulses from chemoreceptors, even though touch is not normally the effective stimulus for the proboscis extension reflex.

Such a cooperation between sensory systems should make possible an economy in terms of the number of receptors of each type necessary; a limited amount of cross summation between associated stimulus modalities would increase the effectiveness of each stimulus. A high level of spontaneous activity in unstimulated receptors should increase similarly the effect of stimulation of a portion of the receptor population.

Electrophysiological observations, both those relating to insect mechanoreceptors and those reported here for chemoreceptors, show a background of spontaneous activity. DDT increases the effectiveness of this background by converting single nerve impulses to trains of impulses. The more frequent afferent impulses

which result from normal stimulation are also made repetitive by the action of DDT. Opportunities for summation both within and among modality groups are thus increased.

Observation of DDT-poisoned insects corroborates this interpretation. Shortly after exposure to the insecticide certain response thresholds of DDT-sensitive individuals become lower. A decided hyperexcitability develops, and this gives way to random reflex activity as, presumably, more and more receptors become unstabilized. Uncoordinated locomotor movements and proboscis extension and retraction are characteristic symptoms of this stage of poisoning. In DDT-sensitive flies the effect of DDT on individual receptors is irreversible.

Summary

1. DDT appears not to stimulate sensory endings, but makes them capable of repetitive discharge following stimulation with toluene and benzene.

2. Pretreatment with DDT lowers about nine-fold the sucrose acceptance thresholds of DDT-sensitive houseflies and blowflies. Sucrose thresholds of resistant flies are unaltered.

3. Pretreatment with DDT does not change salt or alcohol rejection thresholds, either against sucrose or water.

4. Under certain conditions clean smooth surfaces can evoke feeding behavior similar to that in response to acceptable chemicals.

5. DDT-sensitive houseflies, given a choice, spend a greater amount of time on DDT-treated areas. Resistant flies do not. Surfaces treated with DDE and 1,1-bis (p-chlorophenyl) ethane are not discriminated from control surfaces.

6. It is concluded that acceptable and unacceptable compounds are perceived through different sets of receptors.

7. The proboscis extension reflex is not controlled by chemoreceptor activity alone, but also by tactile stimuli.

8. The failure of DDT to affect reflex behavior of this strain of DDT-resistant houseflies is not due to failure to penetrate the cuticle, but must be due to some mechanism intrinsic to the nervous system.

LITERATURE CITED

Dethier, V. G., 1953. Summation and inhibition following contralateral stimulation of the tarsal chemoreceptors of the blowfly. *Biol. Bull.*, **105**: 257–268.

Dethier, V. G., and L. E. Chadwick, 1948. Chemoreception in insects. *Physiol. Rev.*, **28**: 220–254.

Finney, D. J., 1952. Probit analysis. University Press. Cambridge. Second edition.

Pratt, J. J., Jr., and F. H. Babers, 1953. Sensitivity to DDT of nerve ganglia of susceptible and resistant house flies. *J. Econ. Entomol.*, **46**: 700–702.

Roeder, K. D., and Elizabeth A. Weiant, 1948. The effect of DDT on sensory and motor structures in the cockroach leg. *J. Cell. Comp. Physiol.*, **32**: 175–186.

Roeder, K. D., and Elizabeth A. Weiant, 1951. The effect of concentration, temperature, and washing on the time of appearance of DDT-induced trains in sensory fibers of the cockroach. *Ann. Ent. Soc. Amer.*, **44**: 373–380.

Roys, C. C., 1954. Olfactory nerve potentials a direct measure of chemoreception in insects. *Ann. N. Y. Acad. Sci.*, **58**: 250–255.

LOSS AND GAIN OF HEAT-TOLERANCE BY THE CRAYFISH

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It has been well established that a fish becomes increasingly tolerant of heat as it becomes acclimatized to higher temperatures within the range of thermal tolerance of its species, and that its acclimatization to low temperature entails a loss of heat-tolerance (Doudoroff, 1942; Brett, 1944, 1946). Inasmuch as aquatic arthropods are exposed to the same fluctuations in environmental temperature as fish, one might reasonably expect to find that they, too, are capable of gaining and losing heat-tolerance. The matter has not been explored as thoroughly for aquatic arthropods as for fish, however, and there are but few instances in which it is clear that individual animals have gained or lost heat-tolerance, *i.e.*, that they have undergone "physiological" acclimatization, as the term is used by Prosser *et al.* (1950). It is true that aquatic arthropods from warm waters have been found to be more heat tolerant than related species, or even members of the same species, inhabiting cooler waters (Mayer, 1914; Huntsman and Sparks, 1924; Fox and Wingfield, 1937; Mason, 1939; Whitney, 1939; Park, 1945; Walshe, 1948; Marlier, 1949; Bovbjerg, 1952), but in most cases the differences in tolerance can be attributed as well to selection as to physiological acclimatization (Fox, 1939).

With one possible exception, the literature bearing directly upon the problem of physiological acclimatization among aquatic arthropods indicates that they do become increasingly tolerant of heat as their environmental temperatures are increased. Huntsman (1924) showed that lobster larvae raised at temperatures between 20 and 25° C. were more heat resistant than those raised at 15°, Edwards and Irving (1943) reported the thermal death point of the sand crab, *Emerita talpoida,* to be about 10° higher in summer than in winter, and Marlier (1949) found indications that the lethal temperature of larvae of the caddis fly, *Hydropsyche angustipennis,* increased from 31° in the spring to 32° in early summer. Furthermore, Bovbjerg (1952) observed that two species of crayfish, *Orconectes propinquus* and *Cambarus fodiens,* became increasingly tolerant of temperatures between 34 and 35° as the advancing season warmed their habitats, or after they had been maintained in warm water in the laboratory for five or six weeks. The possible exception was reported by Whitney (1939), who found nymphs of the mayfly, *Baetis rhodani,* to be no more heat tolerant after 40 hours at 15° than controls maintained between 10 and 11°. In view of Brett's (1946) experience with the goldfish, however, in which it was shown that the development of an increased heat-tolerance required a latent period of from one to seven days, the latent period being longer the lower the acclimatization temperature, it may be that the absence of acclimatization in *Baetis* was more apparent than real. It seems quite possible that more than 40 hours of acclimatization were required to increase the heat-tolerance enough to be detected by the method employed.

Less seems to be known about the loss of heat-tolerance by aquatic arthropods. Aside from the foregoing observations on the sand crab and crayfish, in which the seasonal gain of tolerance suggests a previous seasonal loss, no literature bearing directly upon this matter has been found.

Relatively little appears to have been published about the temperature relations of the crayfish. The mere fact of its occurrence in the northern part of the United States shows, of course, that it can survive over a range of temperatures extending from about 0 to more than 30°, and direct observations by several authors bear this out. Crayfish are active at temperatures between 4 and 6° (Van Deventer, 1937) and under two inches of ice (Bovbjerg, 1952). They will live at 3° in the laboratory (Kyer, 1942), and adult females have been found to survive freezing overnight (Langlois, 1937). Bovbjerg (1952) has recorded that they occur in ponds that reach 30°, and that acclimatized crayfish have remained alive after a period of seven days at 34 to 35° in the laboratory. In connection with the present study it was observed that adult crayfish were abundant in a shallow river of which the surface temperature at any rate reached 32°. In view of its convenient size, longevity, availability and wide range of thermal tolerance, the temperature relations of the crayfish are of considerable interest not only to the ecologist but also to the physiologist concerned with the problem of explaining the mechanism of acclimatization to temperature. The experiments to be described in this paper were undertaken to provide further information on the thermal relations of the crayfish, especially in connection with the loss and gain of heat-tolerance.

I wish to thank Dr. T. H. Langlois, Director of the Stone Institute of Hydrobiology, for making the facilities of the Institute available for this study, Dr. W. F. Hahnert, of Ohio Wesleyan University, for identifying the species of crayfish, and Drs. C. K. Weichert and W. A. Dreyer, of the University of Cincinnati, for criticism of the manuscript.

Material and Methods

The crayfish, *Orconectes rusticus* (Girard), were collected in July of 1953 and 1954 from Sugar Creek, a small tributary of the Portage River, which enters western Lake Erie at Port Clinton, Ohio. The stream temperatures at the times of collection varied between 22 and 26° C. The animals were maintained at Stone Institute in shallow tanks of running lake water, the temperature of which ranged betwen 22 and 26° (usually between 23 and 24°), and the pH between 8.4 and 8.7 (usually between 8.5 and 8.6). The crayfish seemed to remain in good health under these conditions—they fed eagerly on mussel, fish, bread and lettuce, many molted, the mortality was low, and those not removed for use in the experiments were alert and lively after about six weeks.

Aside from several dozen adult crayfish used only to test the effects of size on heat-tolerance, the seven or eight hundred specimens used in the course of the study ranged between 17 and 42 mm. in total length (from the tip of the rostrum to the tip of the telson when straightened out), most of them being between 25 and 35 mm. They were sexually immature, and presumably in their first summer of life (Van Deventer, 1937; Tack, 1941). Groups of from 25 to 100 were taken

at random from the stock tanks and maintained in well-aerated glass aquaria at acclimatization temperatures of 30 ± 0.1, 12 ± 0.5 and $4 \pm 0.5°$. Those held at 30° were warmed to this temperature from that of the stock tank over a period of several hours. Those in the low-temperature baths were transferred directly from the stock tank, it having been found unnecessary to cool them slowly. Care was taken that no metal came into contact with the water in the aquaria. The animals at 30 and 12° were fed bread and lettuce; those at 4° did not feed. About a fourth of the water in each aquarium was replaced every day or so with fresh lake water, previously brought to the appropriate acclimatization temperature and aerated. The crayfish remaining in the stock tanks were used as controls.

The heat-tolerance of control and experimental animals was studied by testing their survival for 12 hours or longer at 33, 34, 35, 36 and 37°, it having been ascertained in preliminary tests that this range of temperatures spanned the 12-hour median heat-tolerance limits (Doudoroff, 1942; Brett, 1944) of the several acclimatized groups. The test temperatures were maintained to within 0.1°. The animals were tested singly in 10-oz. wide-mouth bottles or in groups of 5 to 10 in one-liter Erlenmeyer flasks, these vessels being held at the test temperatures in constant temperature baths. Each bath held a number of such vessels, so that it was possible to test as many as 40 crayfish simultaneously at one temperature. In preparation for a test, aerated fresh lake water was brought to the temperature of the acclimatization bath from which the animals were to be taken. One hundred ml. of this water were then poured into each bottle, or one liter into each flask, so that at least 100 ml. of water were provided for each animal being tested. The crayfish were then transferred to the vessels and warmed gradually to the test temperature, the test period being started as soon as this temperature had been reached. Aeration sufficient to maintain an oxygen concentration of at least 80% air-saturation was continued throughout the warming and test periods.

The warming period usually lasted for from 15 to 60 minutes, according to the difference between the acclimatization and test temperatures. This procedure was chosen in preference to that of transferring the crayfish abruptly from the acclimatization temperature to the test temperature because the property under consideration was the ability to survive for 12 hours or longer at the test temperature, rather than that of being able to withstand the shock of a sudden plunge from one temperature extreme to another. A sudden plunge into warm water was found to constitute so massive a shock, especially when the difference between the two temperatures spanned some 25 degrees or more, that the distress of the animals was intense, sometimes enough to cause them to throw off one or both chelae. It is possible, of course, that the period of warming allowed them to gain somewhat in heat-tolerance over that possessed at the acclimatization temperature, but such a gain in tolerance, if it occurred at all, was not great enough to obscure the results. In this connection Brett (1941) has reported that raising the temperature gradually from acclimatization to test temperatures over a period of 15 minutes had no effect on the lethal temperatures of speckled trout.

The condition of each crayfish was observed as it reached the test temperature, and from time to time in the course of the ensuing test period. When no movement of the appendages could be detected, or elicited by prodding with a glass rod, the crayfish was considered to be dead. Death was confirmed in each

case at room temperature after the test, and at this time the length of each animal was measured, the sex determined, and gastroliths sought. Each gastrolith found was measured along its greatest diameter, and its thickness noted, the size serving as an index to the stage of the molt cycle (Scudamore, 1947). As the responses of the crayfish tested in 1954 were practically the same as those of the 1953 collection, the data of the two years have been combined.

Results and Interpretation

1. Heat-tolerance of control animals

Crayfish that had been maintained in the stock tanks for from one to five weeks at temperatures between 22 and 26° survived for not more than 6 hours at 37°, for more than 12 but usually not more than 24 hours at 36°, for at least 10 days at 35°, and for at least 24 days at 34°. Their survival of 12-hour and 24-hour exposures to the three higher temperatures is shown in Table I. Evidently the 12-hour median tolerance limit for these animals was above 36°, but below 37°. When the per cent surviving at each temperature is plotted against the test

Table I

Survival of crayfish taken from temperatures between 22 and 26° and tested at 37, 36 and 35°

Test temperature °C.	Number of tests	Number of crayfish	Crayfish surviving			
			for 12 hours		for 24 hours	
			Number	Per cent	Number	Per cent
37	9	76	0	0	—	—
36	15	109	86	78.9	19	17.4
35	12	78	78	100	73	93.6

temperature (Figure 1), the 12-hour median heat-tolerance limit is seen to have been in the vicinity of 36.4°. That for 24 hours was 35.6°.

Their survival for long periods at 34 and 35° confirms and extends Bovbjerg's (1952) observations. In view of the proximity of these two temperatures to the 24-hour median tolerance limit of the controls, it is of interest that the crayfish molted and fed at 34 and 35°. The small difference between survival- and death-temperatures is quite striking, although not peculiar to the crayfish. It has been observed before in experiments with the sand crab (Edwards and Irving, 1943) and with the greenfish, *Girella nigricans* (Doudoroff, 1942).

2. Loss of heat tolerance

When transferred from the stock tanks to an acclimatization bath at $4 \pm 0.5°$, the crayfish lost the heat-tolerance characteristic of the control group, the loss becoming greater the longer the exposure to low temperature. The 12-hour median heat-tolerance limit fell from 36.4 to 35.3° by the end of the fourth day, to 34.8° by the end of the eighth day, to 34.1° by the end of the twelfth day, and

to 33.5° by the end of the sixteenth day in the cold, as shown in Figure 2. Apparently the loss of tolerance did not begin immediately upon exposure to the cold, however, the tolerance after two and a half days being about the same as the original value. The fact that the minnow, *Pimephales promelas,* shows a similar latent period in the loss of heat-tolerance (Brett, 1944) suggests that this may be a rather general characteristic of animals. The apparent slight increase in tolerance after one day in the cold may have been due to chance, the number of crayfish tested at this point being rather small, but it seems worth noting in this

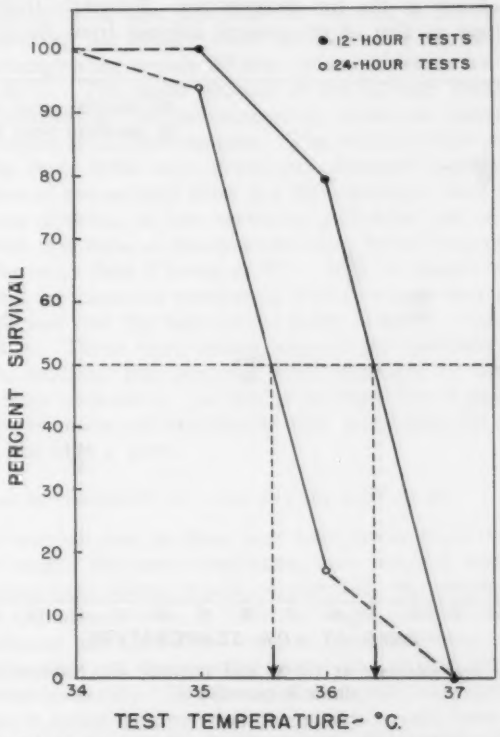

FIGURE 1. Survival at 35, 36 and 37° of crayfish acclimatized to temperatures between 22 and 26° C. The arrows mark the 12- and 24-hour median heat-tolerance limits.

connection that Sumner and Doudoroff (1938) and Doudoroff (1942) have made similar observations on two species of fish. Presumably the crayfish would have continued to lose heat-tolerance, had they been left longer at 4°.

Stock crayfish transferred to 12 ± 0.5° lost their original heat-tolerance in a similar manner, but more slowly. As shown in Figure 2, the 12-hour median tolerance limit had fallen to 35.6° by the end of five and a half days, and to 35.1° by the end of the second week.

3. Gain of heat-tolerance

The heat-tolerance lost during exposure to low temperature was regained quite rapidly when the crayfish were removed from the cold bath. Twenty specimens that had been held at 4 ± 0.5° for thirteen days, long enough for them to have lost their original heat-tolerance by more than two degrees and to have reached a median tolerance limit of about 34° (Fig. 2), were warmed to room temperature (23.5 to 24°) in the course of about two hours and maintained at this temperature for 22 hours. When they were then tested at 36°, 90% lived for 12 hours and 40% survived 24 hours at the test temperature. Evidently their heat-tolerance was at least as great as that of the control animals from the stock tanks, the

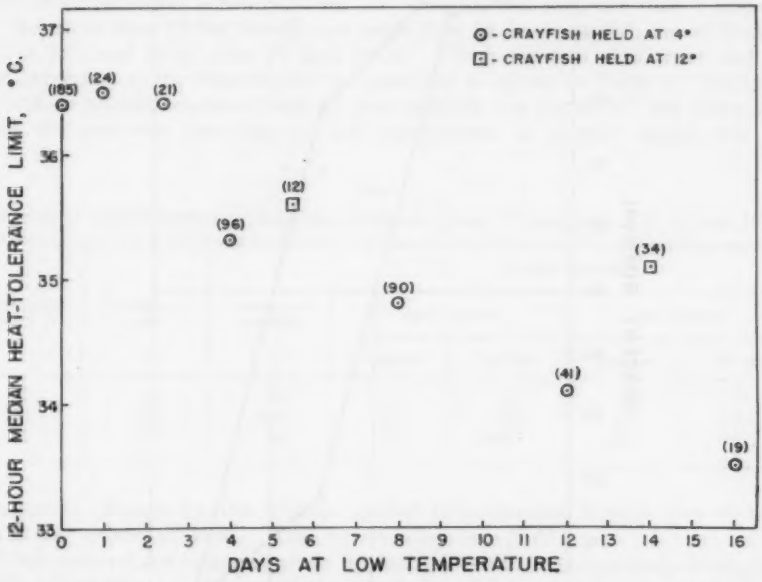

FIGURE 2. Loss of heat-tolerance at 4 ± 0.5 and 12 ± 0.5° C. Numbers of test animals shown in parentheses.

survival of the latter at 36° averaging 78.9% for 12 hours and 17.4% for 24 hours (Table I). Results consistent with these were obtained with six crayfish tested at 35° after one day, and with nine tested at 36° after two days, at room temperature, five of the six and seven of the nine surviving the 12-hour test periods. As in fish (Doudoroff, 1942; Brett, 1944, 1946), the rate of gain of heat-tolerance by the crayfish seems to be considerably higher than the rate of loss, the tolerance lost in about ten days at 4° (after the first two and a half days had elapsed) being recovered in not more than one day.

Upon acclimatization to a higher temperature, the crayfish gained a heat-tolerance somewhat greater than that characteristic of acclimatization to 22–26°.

The 12-hour and 24-hour median heat-tolerance limits of a group of crayfish maintained in an aquarium at 30 ± 0.1° were compared with those of a control group maintained under the same conditions in another aquarium, but at room temperature (between 22 and 25°). Both groups were tested over a three-week period, 20 of each group being tested at the end of each of the first two weeks, and 10 of each group at the end of the third week. The 12-hour median tolerance limit of the 30° animals was 36.6° by the end of the first week and remained at this level for the next two weeks. The corresponding limits for the controls were 36.2, 36.4 and 36.5° after the first, second and third weeks, respectively. The 24-hour limit also was 36.6° at the end of the first week and, like that for 12 hours, did not change from this value during the second and third weeks. The corresponding weekly 24-hour tolerance limits for the controls were 35.5, 35.6 and 35.5°. The slight increase in the 12-hour median tolerance limit is not in itself convincing, but the increase by about one degree in the 24-hour tolerance limit represents a clear-cut gain. The reality of this gain is indicated by the fact that the three tests were consistent, although conducted a week apart. Furthermore, five of the animals from the 30° aquarium lived for more than 12 hours at 37°, one of them, in fact, surviving a 24-hour test at this temperature. It will be recalled that none of the controls taken from temperatures between 22 and 26° lived for more than 6 hours at 37°. It is of interest that continued exposure to 30° did not cause an increase in tolerance over that shown by the end of the first week, and that the limit for 12 hours reached a value no higher than that for 24 hours. These observations suggest the possibility that 36.6° was not far from the maximal heat-tolerance limit attainable by the population from which the collections were made. In view of the high rate of gain of heat-tolerance described earlier, it seems not improbable that acclimatization to 30° was completed in much less than a week.

4. Heat-tolerance in relation to sex, size and the molt cycle

Although all crayfish used in these tests were drawn from the same population and maintained under the same conditions, they were of course not all alike. Males and females were about equally represented, the lengths ranged from 17 to 42 mm., and the animals were at different stages of their molt cycles. About 3% of the specimens had gastroliths large enough to indicate that they were at the molting stage; the rest had smaller gastroliths (about 22%) or none that could be seen macroscopically (75%). As the crayfish were distributed randomly among the tests, it seems unlikely that the results would have been affected by these variables, even if there had existed a relationship between heat-tolerance and sex, size, or the stage of the molt cycle. As a matter of fact, the evidence at hand indicates that no such relationship existed.

Because of the random distribution, males and females, large and small specimens, and representatives of different stages in the molt cycle could be found in each series of tests. The effects of each of these variables could be investigated, therefore, by comparing the animals that survived with those that died at each test temperature. In this manner it was found that as in the sand crab (Edwards and Irving, 1943) and the amphipod, *Hyalella azteca* (Bovee, 1949), sex did not affect the heat-tolerance of the crayfish. It must be borne in mind, of course, that

the specimens were immature. No relationship between size and tolerance could be detected, and the absence of such a relationship is indicated further by the fact that 30 adult specimens, of body lengths between 56 and 82 mm. and with the same history as the control animals in the stock tanks, were neither more nor less heat tolerant than the controls. No inconsistency is seen between these results and reports that larger specimens of the sand crab (Edwards and Irving, 1943) and *Hyalella azteca* (Bovee, 1949) were somewhat more heat resistant than small ones, the methods used in the present study being so different from the others that no direct comparison can be made. Using methods essentially the same as those described in this paper, Doudoroff (1942) and Hart (1947) found no correlation between size and survival time of fish at extreme temperatures. Finally, the stage of the molt cycle evidently had no effect upon heat-tolerance, despite the changes associated with molting and the stress thought to be placed upon the crayfish by the process (Scudamore, 1947). Whether the gastroliths were absent, small, or so large as to indicate that the animals were in molting condition, there was no evidence that their tolerance had been affected. Furthermore, crayfish were found to molt successfully in the acclimatization baths at 12 and 30°, in the course of tests at temperatures as high as 36°, and at room temperature after the period of exposure to the test temperature. They did not molt at 4°.

Discussion

The literature on temperature relations of fish has made it clear that heat-tolerance is so dependent upon acclimatization temperature that expressions of either lethal temperatures or median tolerance limits are incomplete unless accompanied by information on the animal's thermal history. According to the results of the present tests, as well as those described by Bovbjerg (1952), the same can be said of the crayfish. As a consequence, an attempt to compare the heat-tolerance of the crayfish with those of other aquatic animals is hampered by the fact that in most cases the acclimatization temperatures have been different from those used in this study. Such a comparison is limited further by differences among the criteria used in the measurement of heat-tolerance. Whereas 12- and 24-hour median tolerance limits have been sought here, largely so that the results could be related to the more highly developed literature on the temperature relations of fish, most of the data on aquatic arthropods have been expressed in terms either of the death temperatures of animals subjected to ever-increasing temperatures or of the time required for them to die when transferred to constant test temperatures.

Although one gains the general impression that the crayfish is among the more heat tolerant of the aquatic arthropods that have been investigated, direct comparisons are attempted here with but five species (Table II), these having been selected because their acclimatization temperatures were reasonably close to that of the crayfish from the stock tanks. The median tolerance limit given in Table II for *Orconectes rusticus* is that for the 12-hour test period. The figure for *Hyalella* is the temperature at which about half the specimens survived for nearly 11 hours, so that it approximates the 12-hour median tolerance limit. It seems unlikely that increasing the acclimatization temperature by two or three degrees would have increased the median tolerance limit to that of the crayfish. In fish, at any rate, the acclimatization temperature must be increased by several degrees to bring about

an increase of one degree in the median tolerance limit (Fry, Brett and Clawson, 1942; Brett, 1944; Hart, 1947). A temperature of 35° is almost certainly lower than the 12-hour median heat-tolerance limit of Bovbjerg's (1952) acclimatized crayfish, inasmuch as they lived for days between 34 and 35°. On the other hand, 37° is probably somewhat above that of the sand crab, all specimens tested by Edwards and Irving (1943) having died during or shortly after a period of four hours at this temperature. The figures shown for the lobster larvae are the temperatures at which the hearts stopped beating as the animals were warmed at a rate approximating 0.4° a minute; the 12-hour median tolerance limits must have been considerably lower. Although conclusions can be no more than tentative, the sand crab and the three species of crayfish appear to have similar heat-tolerance limits, these being greater by several degrees than those of the lobster larvae and *Hyalella azteca*.

The methods and criteria being more nearly alike, the temperature relations of the crayfish can be compared with those of fish with more confidence. Evidently the crayfish has a heat-tolerance of the same order as that of the more tolerant of

TABLE II

Estimated heat-tolerance limits of several aquatic arthropods related to the 12-hour median heat-tolerance limit of Orconectes rusticus acclimatized to 22–26°

Animal	Acclimatization temperature, °C.	Heat-tolerance limit, °C.	Author
Orconectes rusticus	22–26	36.4	—
O. propinquus	18–28	more than 35	Bovbjerg, 1952
Cambarus fodiens	18–28	more than 35	Bovbjerg, 1952
Lobster, stage 4	25	less than 34.2	Huntsman, 1924
Lobster, stage 5	20	less than 35.4	Huntsman, 1924
Emerita talpoida	20	less than 37	Edwards and Irving, 1943
Hyalella azteca	20	33	Bovee, 1949

the fishes. Of the several dozen species of fresh water fish for which comparable data are available (Fry, Brett and Clawson, 1942; Brett, 1944; Hart, 1947; Black, 1953), the acclimatization temperatures and criteria of tolerance being essentially the same as those used in this study, the brown bullhead (*Ameiurus nebulosus*) and the goldfish (*Carassius auratus*) are among the most tolerant of high temperatures. That crayfish are at least as tolerant as the bullhead is indicated by the fact that bullheads acclimatized to 26° had a lethal temperature (the equivalent of the 12-hour median heat-tolerance limit used here) of 35.3° (Brett, 1944), whereas the corresponding limit for crayfish of similar acclimatization was 36.4°. Apparently the crayfish does not exceed the bullhead in its potential range of tolerance, however, for the 12-hour median tolerance limit of crayfish acclimatized to 30° was about the same (36.6°) as that indicated for the bullhead by Brett's (1944) data. Like the bullhead, the crayfish appears to be less heat tolerant than the goldfish, specimens of which had a 12-hour median heat-tolerance limit somewhat greater than 37° when acclimatized to 28°, according to figures given by Brett (1946). The rather marked similarities between crayfish and fish with respect to loss and gain of heat-tolerance, as described in a preceding section, suggest that the basic

principles of temperature acclimatization are much alike in the two groups, if not identical.

Summary

1. Crayfish (*Orconectes rusticus*) taken from environmental temperatures between 22 and 26° lived for not more than six hours at 37° and for at least ten days at 35°. Their 12-hour and 24-hour median heat-tolerance limits were 36.4 and 35.6°, respectively.

2. Upon being transferred to about 4° and maintained at this temperature, their heat-tolerance, as measured by the 12-hour median tolerance limit, fell to 35.3° after four days, to 34.8° after eight days, to 34.1° after twelve days, and to 33.5° after sixteen days. Heat-tolerance was lost in a similar manner at about 12°, but more slowly.

3. The heat-tolerance lost after thirteen days of exposure to 4° was regained within one day after they had been returned to the original environmental temperature, the rate of gain of heat-tolerance being much higher than the rate of loss.

4. When tested after one week at 30°, the 24-hour median heat-tolerance limit had risen to 36.6° from the initial value of 35.6°. Two more weeks at 30° did not bring about a further increase in this tolerance limit, however.

5. Apparently the heat-tolerance of these crayfish was not affected by sex, size, or the stage of the molt cycle.

6. The results indicate that crayfish are among the more heat tolerant of the aquatic arthropods and fish for which appropriate data are available.

7. The similarities between the temperature relations of crayfish and fish suggest that the basic principles are alike in the two groups.

Literature Cited

Black, E. C., 1953. Upper lethal temperatures of some British Columbia freshwater fishes. *J. Fish. Res. Bd. Can.*, **10**: 196–210.

Bovbjerg, R. V., 1952. Comparative ecology and physiology of the crayfish *Orconectes propinquus* and *Cambarus fodiens*. *Physiol. Zool.*, **25**: 34–55.

Bovee, E. C., 1949. Studies on the thermal death of *Hyalella azteca* Saussure. *Biol. Bull.*, **96**: 123–128.

Brett, J. R., 1941. Tempering versus acclimation in the planting of speckled trout. *Trans. Amer. Fish. Soc.*, **70**: 397–403.

Brett, J. R., 1944. Some lethal temperature relations of Algonquin Park fishes. *Pub. Ont. Fish. Res. Lab.*, No. 63: 1–49.

Brett, J. R., 1946. Rate of gain of heat-tolerance in goldfish (*Carassius auratus*). *Pub. Ont. Fish. Res. Lab.*, No. 64: 5–28.

Doudoroff, P., 1942. The resistance and acclimatization of marine fishes to temperature changes. 1. Experiments with *Girella nigricans* (Ayres). *Biol. Bull.*, **83**: 219–244.

Edwards, G. A., and L. Irving, 1943. The influence of temperature and season upon the oxygen consumption of the sand crab, *Emerita talpoida* Say. *J. Cell. Comp. Physiol.*, **21**: 169–182.

Fox, H. M., 1939. The activity and metabolism of poikilothermal animals in different latitudes. *Proc. Zool. Soc. Lond., Ser. A*, **109**: 141–156.

Fox, H. M., and C. A. Wingfield, 1937. The activity and metabolism of poikilothermal animals in different latitudes. *Proc. Zool. Soc. Lond., Ser. A*, **107**: 275–282.

Fry, F. E. J., J. R. Brett and G. H. Clawson, 1942. Lethal limits of temperature for young goldfish. *Rev. Canad. de Biol.*, **1**: 50–56.

Hart, J. S., 1947. Lethal temperature relations of certain fish of the Toronto region. *Trans. Roy. Soc. Canada, V*, **41**: 57–71.

HUNTSMAN, A. G., 1924. Limiting factors for marine animals. 2. Resistance of larval lobsters to extremes of temperature. *Contr. Canad. Biol., N. S.*, **2**: 91–93.

HUNTSMAN, A. G., AND M. I. SPARKS, 1924. Limiting factors for marine animals. 3. Relative resistance to high temperatures. *Contr. Canad. Biol., N. S.*, **2**: 97–114.

KYER, D. L., 1942. The influence of the sinus glands on gastrolith formation in the crayfish. *Biol. Bull.*, **82**: 68–78.

LANGLOIS, T. H., 1937. Further observations on the habits of the crayfish, *Cambarus rusticus* Girard. *Trans. Amer. Fish. Soc.*, **66**: 275–276.

MARLIER, G., 1949. Relation entre température léthale et habitat normal chez les larves de trichoptères. *C. R. Soc. Biol.*, **143(1/2)**: 100–101.

MASON, I. L., 1939. Studies on the fauna of an Algerian hot spring. *J. Exp. Biol.*, **16**: 487–498.

MAYER, A. G., 1914. The effects of temperature upon tropical marine animals. *Carnegie Inst. Wash., Dept. Mar. Biol., Tortugas Lab.*, **6**: 1–24.

PARK, T., 1945. A further report on toleration experiments by ecology classes. *Ecol.*, **26**: 305–308.

PROSSER, C. L., F. A. BROWN, JR., D. W. BISHOP, T. L. JAHN AND V. J. WULFF, 1950. Comparative animal physiology. W. B. Saunders Company, Philadelphia.

SCUDAMORE, H. H., 1947. The influence of the sinus glands upon molting and associated changes in the crayfish. *Physiol. Zool.*, **20**: 187–208.

SUMNER, F. B., AND P. DOUDOROFF, 1938. Some experiments on temperature acclimatization and respiratory metabolism in fishes. *Biol. Bull.*, **74**: 403–429.

TACK, P. I., 1941. The life history and ecology of the crayfish, *Cambarus immunis* (Hagen). *Amer. Mid. Nat.*, **25**: 420–466.

VAN DEVENTER, W. C., 1937. Studies on the biology of the crayfish *Cambarus propinquus* Girard. *Ill. Biol. Monogr.*, **15**: 1–67.

WALSHE, B. M., 1948. The oxygen requirements and thermal resistance of chironomid larvae from flowing and still waters. *J. Exp. Biol.*, **25**: 35–44.

WHITNEY, R. J., 1939. The thermal resistance of mayfly nymphs from ponds and streams. *J. Exp. Biol.*, **16**: 374–385.

THE MOLTING CYCLE OF THE SPINY LOBSTER, PANULIRUS ARGUS LATREILLE. II. PRE-ECDYSIAL HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN THE HEPATO-PANCREAS AND INTEGUMENTAL TISSUES[1]

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The external manifestations of an approaching molt and of molting itself are the result of more basic physiological phenomena related to growth. Growth in the arthropods is markedly conditioned by the properties of the exoskeleton, which are in turn conditioned by the epidermis and other tissues. In the Crustacea, as in other arthropods, growth is cyclical, periods of comparative rest alternating with periods of activity. These cyclic periods of growth are accompanied by cyclical changes in the epidermis, sub-epidermal tissues and the hepatopancreas. The present paper is a study of the pre-ecdysial histological and histochemical changes in the integumental tissues and the hepatopancreas of *Panulirus argus* Latreille.

Materials and Methods

Animals

Male and female spiny lobsters ranging in carapace length from 80–89 mm. were obtained and handled as previously described (Travis, 1954).

Designation of stages in the molting cycle

In a study of the molting cycle, some method of designating the essential stages is necessary. Two main methods have been resorted to, namely: 1) indication of actual time periods, and 2) indication of morphological characters of skeleton and tissues. The first of these methods offers certain advantages. By this method, it is possible to determine the length of the intermolt periods in each size group and to determine the duration of existing morphological characters of the skeleton at each season of the year. In this manner any animal in the laboratory can, from its previous laboratory record, be placed in the proper stage of the molting cycle either by days following molt or by the morphological characters of the skeleton. The second method consists of breaking down the molting cycle into four major stages, A, B, C, D, and subdividing each of these stages, according to Drach (1939).

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Its major advantage is that it provides a means of grouping animals into the major stages of the molting cycle without reference to the interval of time over which such stages extend. Another advantage of Drach's method is that, regardless of age or size animals of corresponding stages can be compared even though the duration of these stages varies with size and season.

Both methods should be used since they are supplementary and necessary for interspecific comparison. The following time intervals for the molting cycle of *Panulirus* refer to animals of 80–89 mm. carapace length. The length of the entire intermolt period in the summer months is usually 65–70 days (Travis, 1954).

Stage A—Stage immediately following molt. The exoskeleton is of the consistency of a soft membrane. The animals do not feed. The duration is about 24 hours.

Stage B—Preliminary hardening of the skeleton occurs, which attains the consistency of parchment. The carapace is rigid in certain regions while the branchiostegites remain soft. No feeding occurs. The duration is from one day postmolt through the fifth or sixth day.

Stage C—The skeleton is entirely hardened but continues to thicken during a good portion of the period. This is a period of active feeding and is the longest of

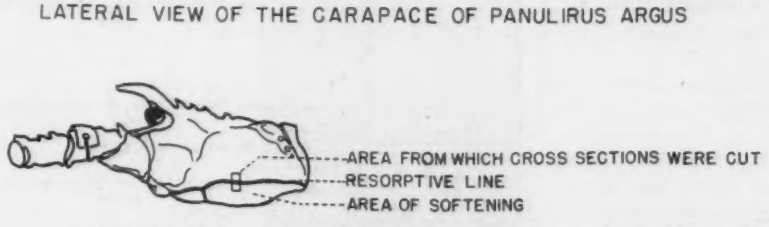

LATERAL VIEW OF THE CARAPACE OF PANULIRUS ARGUS
AREA FROM WHICH CROSS SECTIONS WERE CUT
RESORPTIVE LINE
AREA OF SOFTENING

FIGURE 1. Lateral view of the carapace of *Panulirus* to show the area from which sections of the integument were cut.

the cycle, lasting from approximately the seventh to the fifty-first day following molting, or a total of about forty-four days. During late Stage C, the membranous layer and the principal layer are completed. The term "intermolt animal" will be applied to animals with a fully developed skeleton, a condition which falls approximately midway in days between two ecdyses, *i.e.*, 28–35 days following one molt and preceding the next.

Stage D—The new future skeleton is progressively constructed under the old, while the old is gradually broken down by resorption of both mineral and organic constituents. The period lasts ten to fourteen days during which the animals do not feed.

Histological and histochemical methods

For the histological and histochemical studies pieces of skeleton were removed from the carapace of *Panulirus* (Fig. 1) approximately five, three, and one days before a molt. Also, pieces of integument were removed on each of eight consecutive days following molt (a study of which will be reported in a subsequent paper) and from intermolt animals (late Stage C). By removal of pieces of skele-

ton during the pre-ecdysial period (late Stage D) the extent of the resorption in the endocuticle could be detected.

The right posterior lobe of the hepatopancreas was removed at approximately three days and one day before molt, on each of seven consecutive days following molt, and from animals in late Stage C.

All integumental tissues were embedded in celloidin and cut at 10 μ. The posterior lobe of the hepatopancreas was embedded in paraffin and cut at 8 μ. One to three animals were used to represent each of the days mentioned above. Portions

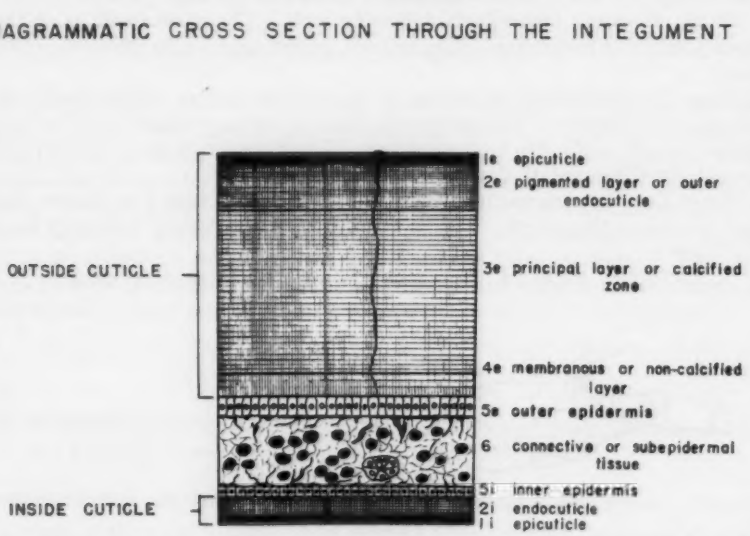


FIGURE 2. Diagram of a section through the integument of the branchial region (late Stage C). Note outside cuticle consisting of four distinct layers with its epidermis and inside cuticle consisting of two distinct layers with its epidermis. The inside cuticle borders the outer periphery of the gill chamber. The spongy sub-epidermal connective tissue is sandwiched between the outer and inner epidermis. The large black oval cells represent reserve cells.

of skeleton and hepatopancreas fixed in Helly's and alcoholic Bouin's fluid were stained by the following methods:

1. Mallory's triple stain.
2. Periodic acid-Schiff (PAS) of McManus, as described by Lillie (1948).
3. Bensley and Bensley's method (1938), demonstrating mucins by means of toluidine blue. The thiazine dyes have three absorption bands: alpha, beta, and gamma. The alpha form is orthochromatic (blue), whereas beta metachromasia (violet) can be caused by highly polymerized carbohydrates or by phosphate-containing compounds. Substances which give gamma metachromasia (red or pink) are usually acid mucopolysaccharides (Pearse, 1953). Since chitin is considered to be a neutral mucopolysaccharide (Meyer, 1938) and is closely associated with protein in the arthropod cuticle, since there is no real histochemical distinction

possible between neutral mucopolysaccharides and mucoprotein (Pearse, 1953), and since in early stages of skeletal deposition gamma metachromasia is exhibited, the pink color produced by the dye probably indicates the presence of muco- or glycoproteins.

For the detection of calcium deposits, portions of the skeleton were fixed in nine parts of 95% alcohol and one part of 40% formaldehyde and were stained with the following:

1. Mallory's triple stain.
2. Schmorl's purpurin (Lillie, 1948).
3. Alizarin red S (Manigault, 1939).
4. Von Kossa's method (Lillie, 1948). Before following this procedure, tissues were washed in 5% aqueous KNO_3 for five minutes or more to remove some of the chloride present.
5. Microincineration (Scott, 1933) was used to confirm the presence of calcium deposits detected by the stain mentioned above.

The hepatopancreas was also fixed in formol-alcohol and was stained with Mallory's triple and Alizarin red S, the latter stain being used to detect calcium.

Portions of the skeleton and hepatopancreas were fixed in cold 80% alcohol, and alkaline phosphatase was determined by the method of Gomori (1941). Control sections (Fig. 32) were made, using distilled water without added substrate during incubation.

Portions of the hepatopancreas which were fixed in 10% neutral formalin were embedded in carbowax (method of Blank and McCarthy, 1950), cut at 10 and 15 μ and stained for lipids with Sudan black B.

In sections of exoskeleton from premolt animals where the resorptive line becomes apparent, the folding, and in some cases the loss of the old cuticle made detection of this line impossible even in celloidin sections. Therefore, sections made by hand or whole mounts were used to study resorption in this area.

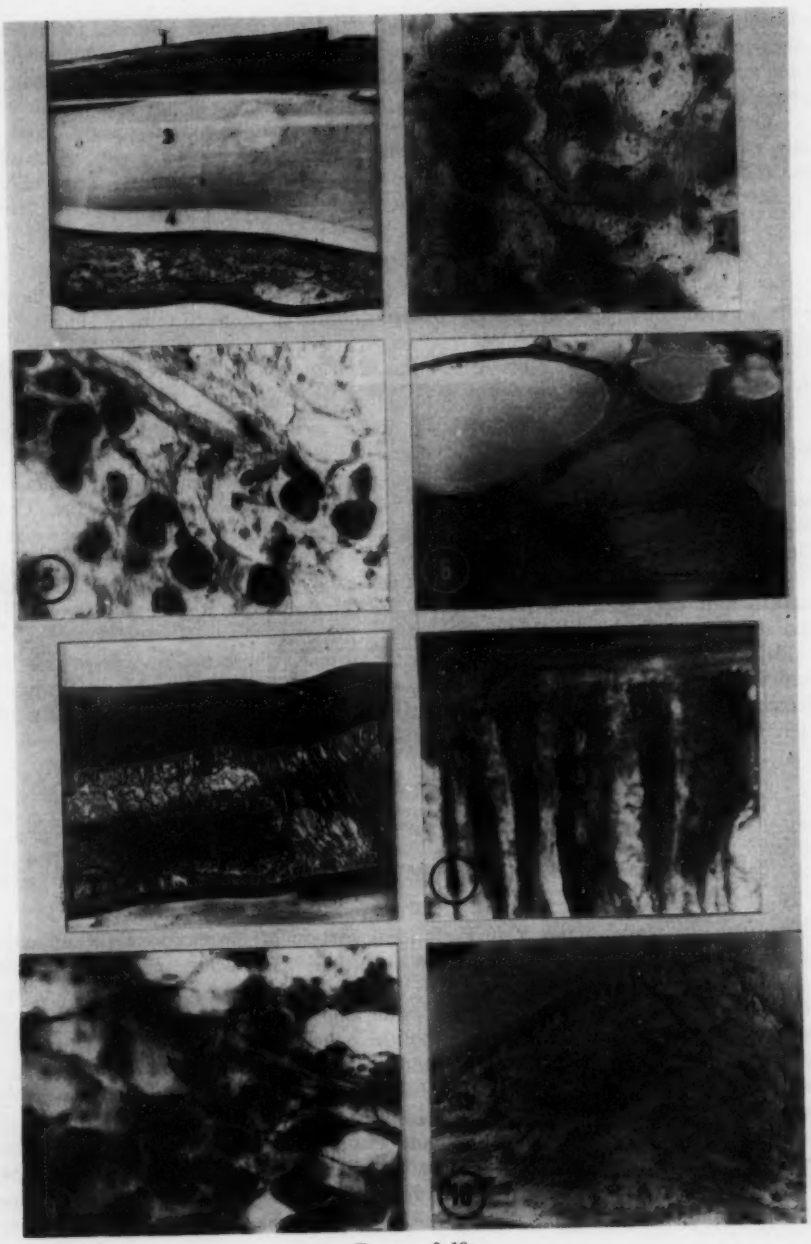
Observations

A. The intermolt animal (late stage C)

1. *The integument and integumental tissues*

a. *Tissues*

In pieces of exoskeleton from the lateral portion of the carapace the attached tissues are bordered by two integuments or cuticles as indicated in Figures 2 and 3. The inner integument, which forms a boundary for the gill chamber, is approximately 1/20 the thickness of the outer one (Fig. 3). Underlying each of these integuments is a layer of columnar epidermal cells with centrally located nuclei, and between these two layers is sub-epidermal connective tissue of a loose spongy type (Figs. 2, 7). Large oval reserve cells (Figs. 4, 5, 9), which Cuénot (1893) called "protein reserve cells" in *Astacus fluviatilis* and which resemble Type 1 Leydig cells (Kükenthal, 1926–1927) and Langer's vesicles of Mollusca (Bronn, 1896–1897), constitute by far the most predominant type of cells in this tissue. They appear as vesicular cells with a capsule-like envelope of cytoplasm containing a peripheral nucleus. These reserve cells vary in appearance during the molting



FIGURES 3–10.

cycle, binding or storing large amounts of polysaccharide material and calcium (Travis, 1951a). In the intermolt state the cells apparently store only polysaccharide (Fig. 4), whereas following molt they store calcium as small or large spheres and take on a "mulberry" appearance (Fig. 5). They may store fat as well. Although no material was fixed for the determination of the presence of lipid in the integumental tissues, similar cells in the connective tissue of the hepatopancreas contain much lipid. Cuénot (1893) described cells in the connective tissue of *Palinurus vulgaris* which stored large quantities of fat. His drawings indicate that these "fat cells" are similar in every respect to the reserve cells.

On first examination of the sub-epidermal tissues, it would appear that the reserve cells vary in number during various stages of the molting cycle, but this is not the case. When binding or storing reserves they are greatly increased in size, and their boundaries and nuclei are easily observed, especially with Helly's fixative and the PAS method. During periods when reserves are depleted, for example in late Stage C, many cells decrease in size and become clear and vesicular. Then cell boundaries are difficult to determine, but PAS and toluidine blue bring them out successfully.

In addition to the reserve cells (Type 1) there are: very small spindle cells with branching processes, *i.e.* Leydig cells, Type 2; rectangular cells (Fig. 6) which surround the walls of arteries, *i.e.*, Leydig cells, Type 3 (Kükenthal, 1926–1927); and amoebocytes in the blood spaces and channels of the connective tissue.

Tegumental glands were occasionally observed in the area from which these sections were cut. Collagen-like fibers are found throughout the connective tissue. At intervals within this tissue, the basal ends of some of the epidermal cells from each integument become long and attenuated and extend from one cuticle to the other (Fig. 7). The tonofibrillae (Fig. 7) which traverse the length of the epidermal cells are in close proximity to parallel collagen-like fibers which probably strengthen these supporting epithelial colonnades (Fig. 7) described by Vitzou (1882) in *Astacus fluviatilis* and *Homarus vulgaris*.

FIGURE 3. Photomicrograph of the integument of an intermolt animal (late State C). The four layers of the outer cuticle are represented by numbers. 1, epicuticle; 2, pigmented zone; 3, principal or calcified zone; 4, membranous layer. Note that outer integument is about 20 times thicker than inner cuticle. 90 ×.

FIGURE 4. Large, oval reserve cells (Leydig cells, Type 1) of the connective tissue with peripheral nuclei, one of which is indicated by arrow. Above arrow, collagen-like fibers found throughout the connective tissue. The small granules are glycogen (late Stage C). 450 ×.

FIGURE 5. The reserve cells take on an irregular "mulberry" appearance and their contents become divided up into small spheres as they store calcium following molt. 430 ×.

FIGURE 6. Large rectangular cells which surround the walls of arteries (Leydig cells, Type 3). The numeral (6) is within the lumen of an artery. 760 ×.

FIGURE 7. Colonnades of support, as indicated by arrow, are found at intervals throughout the connective tissue. 80 ×.

FIGURE 8. Tonofibrillae (T) which traverse the length of the epidermal cells. Arrow points to a pore canal, a protoplasmic extension of one of the epidermal cells. 1000 ×.

FIGURE 9. Photomicrograph showing very small amount of glycogen in the integumental tissues of an intermolt animal (late Stage C). Note small number of granules (a few are indicated at the tip of the arrow) localized between the reserve cells of the connective tissue. 450 ×.

FIGURE 10. Nuclei and cytoplasm of the integumental tissues are almost devoid of alkaline phosphatase during late Stage C. Nuclei indicated at tip of arrow show a positive reaction because of the presence of calcium. 80 ×.

b. *The Integument and its composition*

The outer integument, about 750 μ thick, consists of four distinct layers (Figs. 2, 3):

1. The most external layer, a thin epicuticle, is divided into two portions and is about 11 μ thick. It is composed of a tanned lipoprotein, and is impregnated with calcium salts.

2. A thicker layer, approximately 200 μ, is hardened by quinones as well as by calcium salts. This is called the pigmented layer of the outer endocuticle.

3. The third layer, about 460 μ in total thickness, is heavily calcified but non-pigmented and is called the principal layer or the calcified zone.

4. The fourth layer, approximately 90 μ in thickness, is non-calcified, non-pigmented, and is in direct contact with the underlying epidermis. This is called the membranous layer (Drach, 1939) or the non-calcified layer.

The thin inside cuticle next to the gill chamber is composed of two layers, a very thin epicuticle, and a uniformly staining endocuticle (Fig. 2).

Both the epicuticle and pigmented zone are formed before the molt. Drach (1939), therefore, prefers to call them pre-exuvial layers. He calls the principal layer and the membranous layer post-exuvial layers because of their formation after molt.

The basic components of the crustacean cuticle are, as in insects, chitin and protein, the properties of which may be altered by quinones, impregnation with lipids or calcium salts. Results obtained with toluidine blue would indicate that the principal and membranous layers of *Panulirus* contain a muco- or glycoprotein complex. These give gamma metachromasia (pink) while the pigmented layer shows beta metachromasia (violet). This difference is probably due to the fact that the properties of the protein and closely associated chitin units of the pigmented zone have been changed by quinones. Richards (1952) has similarly found that application of Schiff's reagent for histochemical demonstration of polysaccharides and glyco- or mucoproteins does not always give a positive reaction even though carbohydrate is present. He points out that the carbohydrate in the insect skeleton may be masked wherever sclerotization has occurred.

The horizontal laminations observed in all layers of the cuticle (Fig. 2) with the exception of the epicuticle, have been attributed by Drach (1939) to the rhythmical discharge of secretory material from the epidermal cells. Richards and Anderson (1942), however, suggest that these laminations may be the result of chemical changes in the constituents after secretion.

The vertical cross striae observed in sections of the cuticle are pore canals (Fig. 8), originally associated with protoplasmic extensions of the epidermis. The function of these is not entirely clear. Wigglesworth (1933, 1948) believes that they enable the epidermal cells to act at a distance upon the superficial layers of the cuticle and that hardening of the insect cuticle by quinones is effected through these canals. In *Panulirus argus*, hardening of the pre-exuvial layers by calcification would appear to be effected through these canals following molt (Travis, 1951a, 1951c).

c. *Localization of glycogen, phosphatase and calcium*

During late Stage C, there is little glycogen, phosphatase, and calcium evident in the integumental tissues. Glycogen when observed is sparsely localized between

the reserve cells or the connective tissues (Figs. 4, 9). Alkaline phosphatase appears to be completely lacking in the cytoplasm and nuclei of the integumental tissues. Some calcium, though sparse, is present in all nuclei (Fig. 10), occasionally present in distal ends of epidermal cells, and is infrequently bound in some polysaccharide complex in the few apparent reserve cells. All of the integument with the exception of the membranous layer is fully calcified.

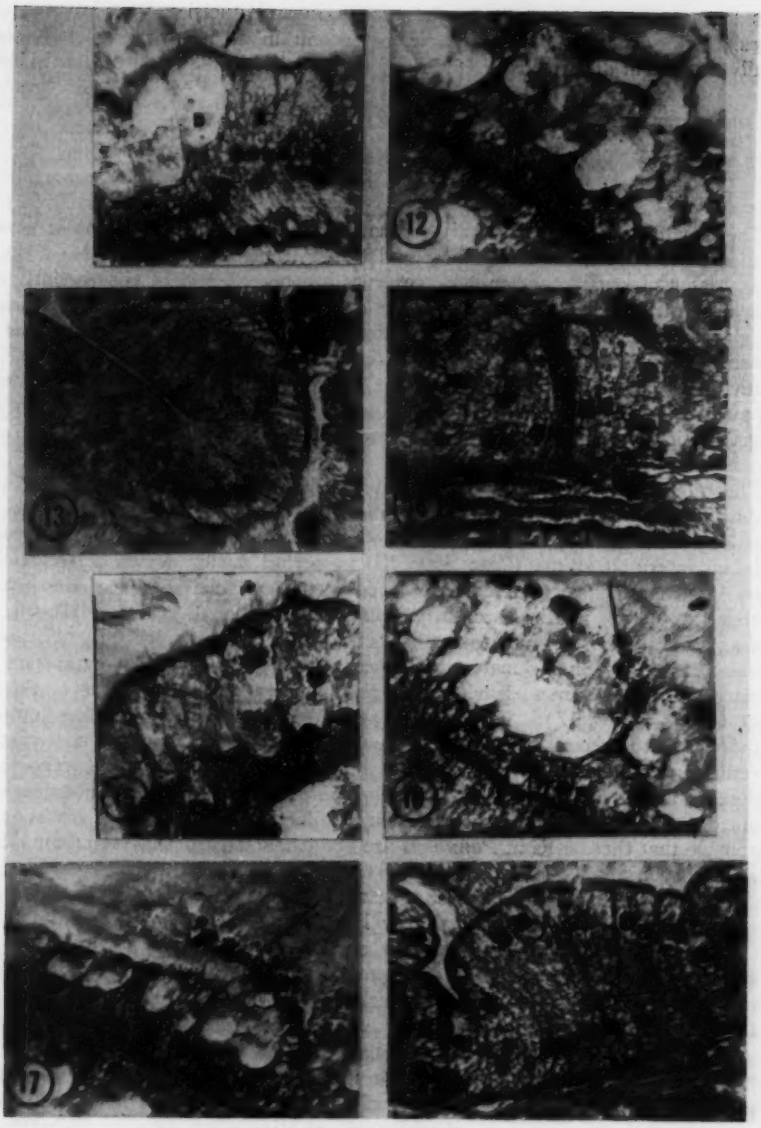
2. The hepatopancreas

a. *Tissues*

The hepatopancreas, a bilateral evagination of the midgut, functions in secreting digestive enzymes and absorbing and transforming food. It is also a major storage depot of organic and mineral reserves in Stage D and is, consequently, the major organ from which these reserves are mobilized when needed by other tissues. The gland itself is composed of innumerable tubules separated from each other by a loose connective tissue, which encloses large blood sinuses and branches of the hepatic arteries. There are collagen-like fibers intermingled between the large oval and vesicular reserve cells (as described in the discussion of the integumental tissues) which constitute the most numerous type of cells in the connective tissue. Amoebocytes are also present.

The tubular tissue proper is composed of columnar epithelium, delimited at its base by a basement membrane and characterized at its distal end (next to the lumen) by a striated border. The nuclei of these epithelial cells are basally located.

In an intermolt animal (late Stage C), a period during which active feeding occurs, the most conspicuous cells observed in the epithelial tissue of the hepatopancreas are large, mature, secretory cells (vesicular or B_2 cells of Hirsch and Jacobs, 1928, 1930). These are swollen, enclosing large vacuoles (Fig. 11), some of which contain stainable material. Their contents plus adjacent cytoplasm are discharged into the lumen, leaving only the basal region and nucleus of the cell intact (Figs. 12, 16, 17). Hirsch and Jacobs (1928, 1930) observed a complete breakdown of the secretory cells in *Astacus leptodactylus, i.e.,* holocrine secretion. Secretion in *Panulirus,* however, occurs in an apocrine rather than a holocrine fashion. The restitution of secretory cells in *Panulirus* is probably not nearly as complex as that observed in Crustacea where holocrine secretion takes place, and it is probable that these cells in *Panulirus* are reconstructed from the remaining basal end. Embryonic cells (E-cells, Fig. 13) predominate at the blind ends of the tubules. Hirsch and Jacobs (1928) pointed out in *Astacus leptodactylus* that these E-cells are followed proximally by B-cells and still more proximally by R-cells (Absorption cells). In *Panulirus* such a distinction is not so clear cut. Secretory cells and what would appear to be absorbing cells (Fig. 11) are frequently observed side by side. Fibrillar cells (Fig. 14) are not numerous, and these plus either absorbing or young secretory cells are scattered throughout the tubular tissue. Mature absorption cells (described in Kükenthal, 1926–1927) are tall columnar cells (Figs. 11, 14, 18, 19) with numerous small vacuoles and with either a basal or central nucleus, depending upon the stage of development. These would appear to be the same as the so-called B_1 cells of Hirsch and Jacobs. Whether the so-called absorption cells (R-cells) are really young secretory cells which have not developed large vacuoles or are special cells limited to absorption alone is not clearly evident.



FIGURES 11–18.

Mature B_2 or secretory cells as well as the tall columnar absorption-type cells contain both fat and glycogen. During Stage D, only the absorption cells contain large numbers of calcospherites. It is not impossible that in their life cycle the same cells perform both functions; absorption in their early stages and secretion in later stages. Certainly the epidermis of the integument performs both functions without the presence of any specialized types within it. In *Panulirus* the predominant hepatopancreatic epithelial cell types from specimens 1–5 days following molt are the long tall columnar cells without large vacuoles, the animal undergoing inanition during this period. Generally, feeding begins on the 6th or 7th day following molting. Because of the difficulty of distinguishing between absorption cells and young secretory cells (B_1-cells) described by Hirsch and Jacobs, and because of the possibility that these cells may perform absorption in their early stage of development and secretion in a later stage of development, the term secretory cell will be applied to those swollen cells containing very large vacuoles at their distal end and undergoing breakdown at the site of the large vacuole (Figs. 11, 12, 15, 16, 17). The name absorption cell will be applied to the tall cylindrical cells which do not contain large vacuoles at their distal end, but which may or may not have small vacuoles, and which may have either a central or basal nucleus (Figs. 11, 14, 18, 19). The presence of a central or basal nucleus may indicate a stage in the maturation of the cell. It will be observed that in Figures 11, 14 and 19 the nuclei of the absorption cells are centrally located, whereas those in Figure 18 are basally located. In the former cases, these cells may be younger than in the latter. In the latter case (Fig. 18), small vacuoles have developed around the calcospherites at the distal ends of the cells, and the nuclei have moved basally. These absorption cells may represent the oldest type before these same cells begin to perform secretion.

FIGURE 11. Portion of the tubular epithelium of the hepatopancreas showing (A) absorption type cells with central nuclei and (B) secretory cells containing large vacuoles. Nuclei in the latter are basally located. Note the striated borders, next to the lumen of the tubule, indicated by arrow. 430 ×.

FIGURE 12. Apocrine breakdown of secretory cells in the hepatopancreas showing ruptured vacuoles. Arrow points to the remnants of a striated border of a secretory cell. Note that the nuclei and basal ends of the secretory cells remain intact. 430 ×.

FIGURE 13. Section through blind end of a tubule of the hepatopancreas showing the numerous young embryonic cells (E-cells). 430 ×.

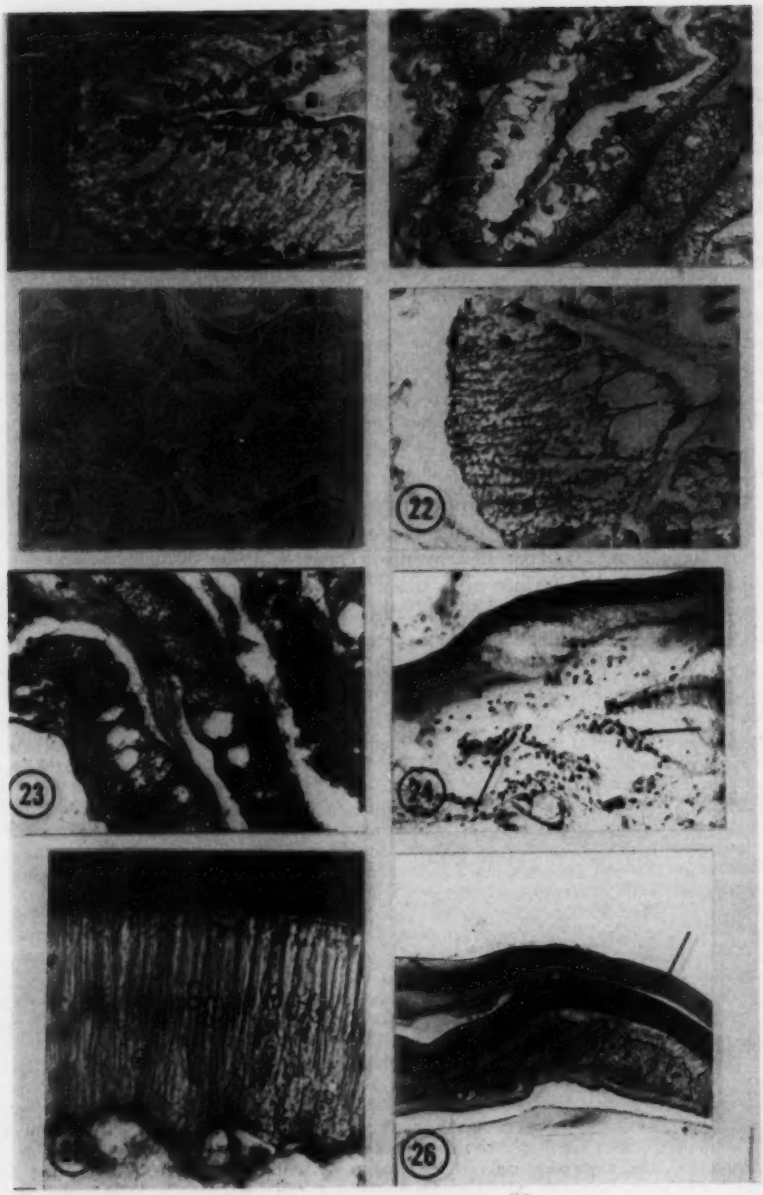
FIGURE 14. The fibrillar cells (F-cells) of Hirsch and Jacobs (1928, 1930). These are rare in the tubular tissue of the hepatopancreas of Panulirus. 430 ×.

FIGURE 15. Mature secretory cells of the hepatopancreas showing striated borders and basal nuclei. Note the secretory material within the vacuoles.

FIGURE 16. Secretory cells of the hepatopancreas which have undergone apocrine breakdown. Note that the basal ends of the cells containing the nuclei remain intact. The secretory granules (at end of arrow) and other contents of the vacuoles are discharged into the lumen of the tubule. Note remnants of striated borders of the secretory cells (to the left of the arrow). 430 ×.

FIGURE 17. Apocrine breakdown in secretory cells of the hepatopancreas. Note discrete secretory granules (at the end of the arrow) extruded into the lumen of the tubule. Basal ends of cells containing nuclei are preserved during secretory breakdown. 430 ×.

FIGURE 18. Old absorption cells of hepatopancreas with basal nuclei and numerous small vacuoles. The arrow points to calcospherites localized at the distal ends of these cells. Section from a premolt animal, fixed with Helly's and stained with Mallory's triple. 430 ×.



FIGURES 19–26.

b. *Localization of glycogen, phosphatase, calcium and lipid*

During late Stage C, the situation in the hepatopancreas is much the same as that observed in the integumental tissues. The few glycogen granules evident in secretory and absorbing cells (Fig. 20) are found at the bases of these cells and around the periphery of reserve cells in the connective tissue. Renaud (1949) demonstrated quantitatively in *Cancer pagurus* that little free glycogen could be detected in the hepatopancreas during most of Stage C because most appears to be utilized in the construction and growth of other tissues.

Alkaline phosphatase, present in small amounts, is localized at the striated borders of the epithelium and around the periphery of small and large secretory vacuoles (Figs. 21, 22). It would appear that the enzyme is strategically localized to participate in resorptive and secretory processes. Though the cell and nuclear membranes, and blood sinuses in the connective tissue give a positive reaction, control sections incubated without substrate indicate that most of this is due to the presence of calcium salts.

Calcium, though very sparse, is observed in all nuclei, cell membranes and in blood sinuses of the connective tissue. A very small amount is bound in complexes of the reserve cells.

Lipid is very abundant in the hepatopancreas during late Stage C. Large and small droplets are present throughout the epithelial tissues (both absorption and secretory cells, Fig. 23), and reserve cells; and they are frequently observed in the lumina of the tubules. Renaud (1949) has similarly observed numerous droplets of fat in the epithelial tissue of *Cancer pagurus* during this stage of the molting cycle.

FIGURE 19. Younger absorption cells, with basal nuclei, of a premolt hepatopancreas showing calcospherites at distal ends of cells. 430 ×.

FIGURE 20. Cross section of a tubule in the hepatopancreas of an intermolt animal (late Stage C) showing small amount of glycogen. Note that glycogen granules, although few in number, are distributed basally in both absorption and secretory cells (arrow). 100 ×.

FIGURE 21. Alkaline phosphatase distribution in the tubules of the hepatopancreas. 80 ×.

FIGURE 22. Portion of a single tubule of the hepatopancreas (late Stage C) at higher magnification to show that alkaline phosphatase is localized in the striated borders (at end of arrow) of the epithelium and around the periphery of small and large secretory vacuoles. 430 ×.

FIGURE 23. Section through hepatopancreas (late Stage C) to show the abundant distribution of lipid in both absorption and secretory cells of a single tubule. 80 ×.

FIGURE 24. A three-day premolt condition of the inner integument and epidermis (late Stage D). The epidermal cells have increased in number by undergoing extensive folding (not shown in this photograph). Some cells have disintegrated (arrows). Amoeboid cells appear in the molting fluid (M). 400 ×.

FIGURE 25. Outer integument and epidermis at three days preceding molt. Note that these outer epidermal cells have resumed an orderly alignment, have become much elongated and, in contrast to the situation observed in the inner integument and epidermis, have already secreted a portion of the new integument. 760 ×.

FIGURE 26. The condition of old and new integuments at about one or two days preceding molt. Note that the pre-exuvial layers of the new skeleton are almost fully formed, while resorption in the old skeleton is almost complete. Arrow to the left shows that about half of the old principal layer has been resorbed and arrow to the right indicates that all of the old skeleton, with the exception of the pigmented layer and epicuticle has been broken down by the molting fluid. These two latter layers are never attacked by the molting fluid. 90 ×.

B. THE PREMOLT ANIMAL (LATE STAGE D)

1. *External signs of the approaching molt*

The first distinct external evidence that *Panulirus* is approaching a molt is the appearance of a resorptive or ecdysial line (Travis, 1951a, 1954) along the branchiostegites. As Richards (1951) points out, along these lines sclerotization, indicated by lack of color, fails to occur. The ecdysial line in *Panulirus* appears as a result of marked resorption of organic and mineral constituents, thus leaving only a thin, translucent membrane. Histochemical tests have indicated that only the epicuticle and a thin mucilagenous layer remain, the latter being either the transformed membranous layer (Drach, 1939) or a transformed portion of the calcified zone. When resorption is complete in the endocuticle and when most of the calcium is resorbed from the epicuticle, some of these lines become weak and break, while others may act as hinges allowing flexibility and expansion of the soft body beneath the area in question (Herrick, 1895; Drach, 1939; Travis, 1951a, 1954).

In the entire area ventral to the resorptive or ecdysial line, less resorption occurs (20% resorption; Travis, 1951a) than in the line. The author calls this "the area of softening." In the summer months premolt animals can be detected as early as 5 days preceding ecdysis by feeling them along the area of softening. The resorptive line itself, however, is not clearly evident before 3–4 days preceding ecdysis.

2. *The integument and integumental tissues*

a. *Tissues and integument*

One of the most marked changes in the integumental tissues of a premolt animal is that observed in the epidermis. Approximately 10–14 days preceding molting in the summer months, detachment of the epidermis from the integument occurs, with a consequent development of a space between the two. This premolting stage was detected inadvertently on a number of occasions when secretory organs were removed from the eyestalks. Ten to fourteen days following epidermal retraction from the skeleton, the animals molted. Shortly following this retraction, growth of this tissue occurs. The mode by which growth is accomplished in the outer epidermis of the branchial region, whether by cell enlargement or by increase in cell number or both, has yet to be determined. The author has been unsuccessful so far in obtaining, for histological purposes, the precise stage at which the outer epidermis retracts and grows. This is difficult to obtain because by the time external signs of an approaching molt are obvious in the branchial region, the outside epidermis is past the retraction and growth stage and has begun to deposit the pre-exuvial layers. The epidermal cells of the inner cuticle increase in number causing a folding of the tissue. Some epidermal cells appear to disintegrate (Fig. 24). This is evident three days preceding molting. It would appear that processes leading to the growth of the epidermis, resorption from the old skeleton, and development of the new inside cuticle, all lag behind the growth of the epidermis, resorption from the old skeleton, and development of the new outside cuticle. This becomes evident from the following. At three days preceding ecdysis, nuclei, possibly of disintegrating cells of the inner epidermis and others of amoeboid cells, become trapped in what appears to be secreted molting fluid (Fig. 24). Yonge (1936) believes that these cells in *Homarus* attack and break down the chitin. At

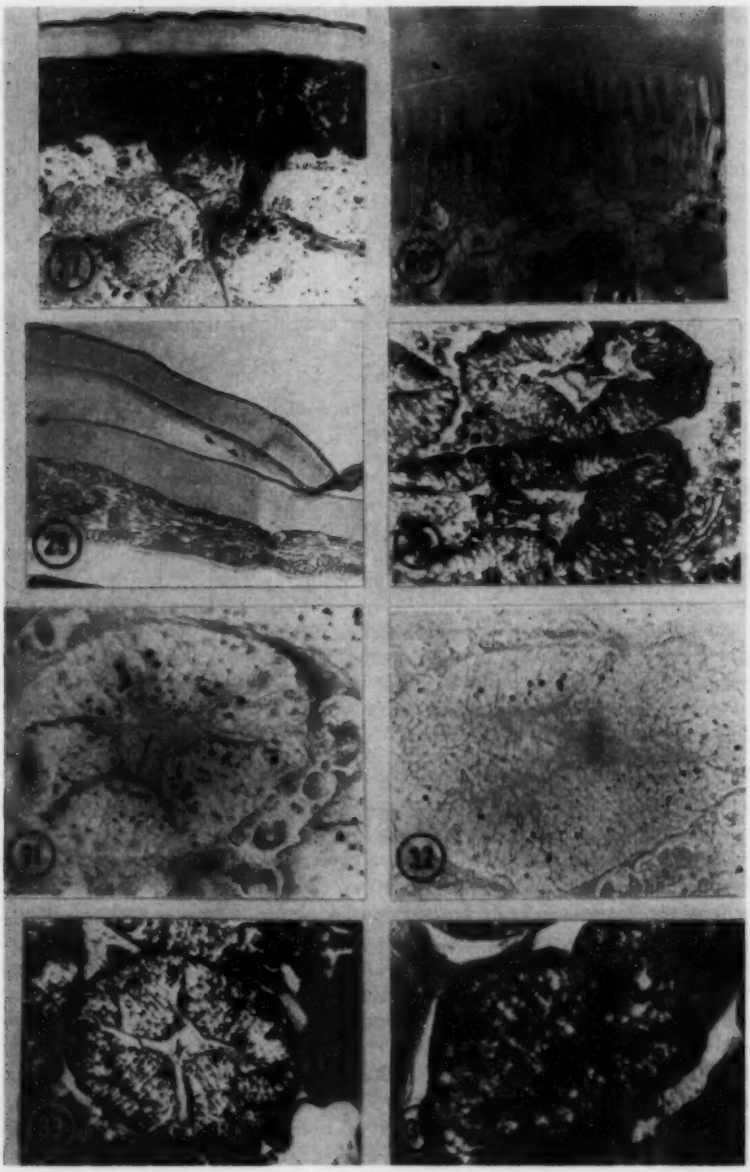
this same time, the epidermal cells of the outside cuticle have resumed an orderly alignment, have become very much elongated, fibrillar in nature, and have already secreted a portion of the new integument (Fig. 25). The new epicuticle of *Carcinus maenas*, shortly after its formation, is hardened by quinones (Krishnan, 1950, 1951). This also appears to be true of *Panulirus*. The thicker of the two pre-exuvial layers, however, becomes hardened by quinones following molt and is then called the pigmented layer. At one to two days preceding molt the old skeleton has thinned considerably (Fig. 26). It will be observed to the left of Figure 26 that almost half of the old principal layer has been resorbed, and to the right of this same figure all of the old skeleton, with the exception of the pigmented layer and epicuticle, has been broken down by the molting fluid. The molting fluid never attacks the latter layers. The immunity of the new epicuticle and pigmented layer to attack by the molting fluid is possibly due to the quinone tanning of the former. The quinone tanning of the epicuticle renders it highly stable and resistant to the enzymes of the molting fluid, but as Richards (1951) has pointed out, there is no adequate explanation of the failure of the newly forming procuticle to be digested by enzymes that must pass through it to reach the molting space.

The almost complete resorption, both mineral and organic, in some of the skeletal areas and partial resorption in others is absolutely necessary for complete separation of the new skeleton from the old and for allowing further thickening of the pre-exuvial layers before ecdysis. Due to resorption from the ecdysial line along the branchiostegites and resorption from an articulating condyle connecting the branchial chamber to the posterior edge of the branchiostegites, lateral expansion of the soft body beneath, and lifting of the old exuvia at molt is allowed (Travis, 1954). As resorption from the old skeleton and building of the new (pre-exuvial layers) progresses in the late premolt period, large numbers of reserve cells become apparent in the connective tissue. These are filled with polysaccharide complexes. This material probably represents not only breakdown products from the old skeleton but reserve substances for the new.

b. *Localization of glycogen, phosphatase and calcium*

During late Stage D, enormous amounts of glycogen are concentrated at the base and throughout the epidermal cells of the inner cuticle (Fig. 27), whereas very little is present in either the outer epidermis (Fig. 28) or the connective tissue. This condition persists until the second day following molt; glycogen then disappears from the inner epidermis for a seven-day period following molt. Little thickening occurs in the inside cuticle after one or two days postmolt, suggesting strongly that this cuticle is formed and completed early. The large amounts of glycogen observed in the inner epidermal cells during late Stage D and Stage A and its disappearance after this integument is completed would suggest that glycogen is a necessary precursor in chitin formation.

Alkaline phosphatase is observed in abundance at the distal ends of the epidermal cells which border on the cuticle (Fig. 29). Krugler and Birkner (1948) have similarly observed a heavy concentration at the distal ends of the epidermal cells of the integument of *Cambarus virilis*. The enzyme appears to be in both reserve cells and cells of Leydig (Type 3). Although these two latter sites are positive in the control sections, they are much lighter in appearance, indicating that the positive



FIGURES 27–34.

reaction of the reserve cells and cells of Leydig (Type 3) is probably due to the presence of calcium and not of the enzyme phosphatase. The enzyme doubtlessly participates in resorptive and secretory processes which are most pronounced at this time.

Although calcium is likewise localized at the distal ends of the outer epidermal cells and in blood sinuses beneath and between these cells, very little is bound in any of the reserve cells during Stage D. While calcium remains in portions of the old integument, *i.e.*, the epicuticle and pigmented layer, no calcification of the new skeleton, in the region of the branchiostegites, occurs until the second day following ecdysis.

3. *The hepatopancreas*

a. *Tissues*

During late Stage D, represented by animals approximately one to three days preceding molt, a marked difference is noted in the tubular epithelium of the hepatopancreas when compared with that of an animal in late Stage C. Likewise numerous reserve cells, filled with lipid and polysaccharide complexes, some of which contain bound calcium, are apparent in the connective tissue. The epithelium lining the tubules becomes high columnar and is composed predominantly of absorption-type cells. One striking difference between the epithelium of a premolt animal and that of an intermolt animal is the appearance of innumerable calcospherites in the distal ends (near the striated border) of the absorbing cells, while none is observed in the fibrillar, and very few are observed in the secretory cells. These calcospherites, spherules of calcium phosphate (previously observed in *Carcinus maenas* by Robertson, 1937), represent calcium salts withdrawn from the old skeleton, some of which are stored in the hepatopancreas during this period prior to molting. These stored calcium salts appear to be used in the hardening of the new skeleton, since calcification proceeds simultaneously with depletion of these stored reserves in the hepatopancreas (to be discussed in a subsequent paper). It is inter-

FIGURE 27. One- to two-day premolt distribution of glycogen in the inner integument. Note the enormous amounts present in the epidermis while very little is evident in the connective tissue. 760 ×.

FIGURE 28. A one- to two-day premolt distribution of glycogen in the epidermis of the outer integument. Note that only very few granules are present. 450 ×.

FIGURE 29. A one- to two-day premolt distribution of alkaline phosphatase in the integumental tissues. The enzyme is heavily concentrated in the distal ends of the epidermal cells which border the integument. 90 ×.

FIGURE 30. The late Stage D distribution of glycogen in the tubular epithelium of the hepatopancreas. Note great concentrations of glycogen in the epithelial tissue and in the lumina of the tubules. 80 ×.

FIGURE 31. The late Stage D distribution of alkaline phosphatase in the hepatopancreas. A single tubule of this tissue is represented. Heavy localization, as indicated by arrow, in the striated borders of the absorbing cells and around the calcospherites. 100 ×.

FIGURE 32. A late Stage D control section of the hepatopancreas. Within a single tubule represented, only the calcium phosphate spherules remain visible following incubation without substrate. 100 ×.

FIGURE 33. The late Stage D distribution of lipid in the tubules of the hepatopancreas. 80 ×.

FIGURE 34. Portion of the epithelium of a single tubule of the hepatopancreas to show the heavy concentration of lipid droplets in the epithelium. 430 ×.

esting to point out that rather large amounts of calcium in the hepatopancreas have been detected by chemical analysis in *Carcinus maenas* by von Schönborn (1912) and Robertson (1937); in *Cancer pagurus* by Paul and Sharpe (1916); in *Maia squinado* by Drach (1939); and in *Hemigrapsus nudus* by Kincaid and Scheer (1952). All of these previous observations have been made on Brachyura. The observations on *Panulirus* indicate that calcospherites are also present in the hepatopancreas of Macrura.

b. *Localization of glycogen, phosphatase, calcium and lipid*

In late Stage D, glycogen, phosphatase, calcium, and lipid are markedly apparent in the hepatopancreas. Glycogen in abundance is observed in the epithelial tissue and even in the lumina of the tubules (Fig. 30). The connective tissue is, however, virtually devoid of it; glycogen is present in blood sinuses and around the periphery of reserve cells. It has previously been observed that during the premolt stage glycogen accumulates in the hepatopancreas of *Astacus fluviatilis* (Bernard, 1879; Vitzou, 1882; Kirch, 1886), *Homarus vulgaris* (Vitzou, 1882), and *Carcinus maenas* (von Schönborn, 1910). In addition to the increase of glycogen in the hepatopancreas before molt, it also appears in the integumental tissues of *Carcinus maenas, Cancer pagurus, Panulirus argus* and *Panulirus japonicus* (Verne, 1924, 1926; Renaud, 1949; Travis, 1951a; and Schwabe *et al.*, 1952). It will be recalled that the integumental tissues of *Panulirus argus* during this same period begin to accumulate large amounts. These stores, initially accumulated by the hepatopancreas, are mobilized and transported to the integumental tissues for the synthesis of the skeleton.

Alkaline phosphatase is heavily concentrated in the striated borders of absorbing cells (Fig. 31). The most striking localization is that observed around innumerable calcospherites, which are sites of calcium phosphate deposition in the distal ends of the absorbing cells (Fig. 31). This very strongly suggests that alkaline phosphatase is directly participating in the deposition of calcium phosphate calcospherites. The secretory cells have few calcospherites and show very little evidence of phosphatase in their striated borders. Thus the enzyme and calcospherites appear to be predominantly localized in absorption-type cells. Though the reserve cells show darkening, control sections indicate that this darkening is due primarily to calcium which is bound in lipid and polysaccharide complexes and not to the enzyme phosphatase. Calcium is also present in blood sinuses within the connective tissue and is localized in cell and nuclear membranes of all cells.

Droplets of fat are found throughout the epithelial tissue (Figs. 33, 34). There is little change over that observed during late Stage C. Where secretory cells are observed, droplets of fat are found throughout these cells, and much of the material in the large vacuoles is lipid. Fat is also abundant in the reserve cells of the connective tissue. Renaud (1949) noted that lipids of the hepatopancreas in *Cancer pagurus* disappear little by little during the fasting period but are still abundant in late Stage D through Stage B, then undergo a marked decrease. The hepatopancreas serves, among other functions, as an important storage depot of fat. Cuénot (1893) was one of the first to indicate this in *Astacus fluviatilis*. In *Cancer pagurus, Lithodes,* and *Homarus* (Paul and Sharpe, 1916) and in *Cancer pagurus* (Renaud, 1949) there is an accumulation of lipids in the hepatopancreas

preceding molt and a gradual disappearance following molt. Likewise Damboviceanu (1932) showed that blood fatty acids increase before molt in *Astacus fluviatilis* and slowly fall following molt and reach normal levels when the skeleton fully hardens.

Thus during Stage D the hepatopancreas becomes an important storage organ for fat, glycogen and calcium reserves. These, as will be pointed out in a subsequent paper, disappear progressively as growth of the integument and other tissues occurs.

Discussion

External signs of an approaching molt in *Panulirus* reflect profound physiological transformations which occur in the tissues of the organism. These changes manifest themselves in the epidermis and sub-epidermal tissues of the integument as well as the epithelial and connective tissues of the hepatopancreas. All of these changes during the pre-ecdysial period are associated with the growth of the organism. The structure restricting the growth in size to certain periods throughout the year is the rigid exoskeleton. A study, therefore, of changes which occur in the integumental tissues, and changes which effect breakdown of the old skeleton and growth of the new, cannot be considered apart from the more general aspects of growth and molting in the arthropods.

During the intermolt period (late Stage C) the skeleton of the branchial region in *Panulirus* is fully hardened and consists of four distinct layers, the epicuticle, the pigmented layer, the principal layer, and the membranous layer. The first three layers are calcified, whereas the fourth is non-calcified. In the Crustacea, the epicuticle and pigmented layer, in addition to being calcified, are hardened by quinones (Dennell, 1947; Kirshnan, 1950, 1951, 1954). In crustaceans as in insects the basic components of the skeleton are chitin and protein which are firmly associated with one another. Trim (1941), Stacy (1943) and Haworth (1946) regard the arthropod cuticle as a mucopolysaccharide because of the firm combination of the carbohydrate containing amino sugars (chitin) with the proteins. Glycogen, phosphatase and calcium are three necessary constituents, therefore, to consider during the breakdown of the old and the growth of the new skeleton.

During late Stage C, no marked changes occur in the hepatopancreas or in the integumental tissues. Few reserve cells are apparent either in the connective tissue of the liver or in subepidermal tissues of the integument. In both areas, such cells contain little carbohydrate, lipid, and calcium. Even though the animals actively feed throughout this period, glycogen is low in both the hepatopancreas and integumental tissues. This is probably the result of its utilization in the growth of other tissue. Renaud (1949), and Schwabe *et al.* (1952), noted similar situations in *Cancer pagurus* and *Panulirus japonicus*, respectively. There is also little evidence of the enzyme phosphatase in the integumental tissues. It is present, however, where active absorption and secretion occur in the hepatopancreas. Calcium is found in the usual locations, *i.e.*, in all nuclei and cell membranes of the integumental and hepatopancreatic tissues and in blood sinuses in the connective tissues. At this period large amounts of fat are stored in the hepatopancreas.

As the pre-ecdysial period (Stage D) is approached, marked changes are observed in the integumental tissues and the skeleton. Most profound of the internal morphological changes are those in the epidermis. In the summer months it is

caused to retract from the old skeleton 10–14 days preceding the molt, so that a molting space develops between the skeleton and retracted tissue. Following this retraction, growth of the epidermis occurs. The mode by which growth occurs has not been observed in the outer epidermis of the branchial region; however, the epidermal cells of the inner integument appear to increase in number causing a folding of the tissue. Such a condition appears three days before molt, indicating that processes which lead to growth of the epidermis, breakdown of the old skeleton, and growth of the new skeleton bordering the gill chamber, lag behind those processes which occur in the outer integument. While growth of the epidermis and breakdown of the old skeleton of the inside integument occur, the epidermal cells of the outer integument have already resumed an orderly alignment, have become very much elongated, and have secreted a portion of the new skeleton (epicuticle and pigmented layer). At one to two days preceding molt, almost all resorption in the old outside skeleton of the branchial region is complete. Mineral and organic resorption from the skeleton—complete in some areas, partial in others—is necessary to free the new skeleton from the old and to allow expansion of the soft body and further thickening of the pre-exuvial layers before ecdysis (Travis, 1954). During this premolt period, when resorption and growth of the new skeleton are occurring simultaneously, large numbers of reserve cells stand out because they are filled with a polysaccharide complex, which may represent either breakdown products from the old skeleton or reserve materials for the new. At this stage, very little calcium is bound to the polysaccharide complex. During the period, large amounts of glycogen are observed in the hepatopancreas. These glycogen granules are localized at the basal and distal ends of the absorbing cells and are observed frequently within the lumina of the tubules. Integumental glycogen comes largely from the hepatopancreas. Since the animals do not feed during this period, much of the glycogen may come from the conversion of fats stored in the hepatopancreas. Large amounts of glycogen are also concentrated throughout the epidermal cells of the inner integument, while very little is evident in either the connective tissue or the outer epidermis. This condition persists until the second day following molt, at which time the glycogen disappears from the epidermis of the inner integument. As the principal layer of the outer integument is deposited following molt, glycogen is observed to accumulate in the outer epidermis. There is a periodic shift of glycogen from the connective tissue to the outer epidermis (Travis, 1951a, 1951c) which probably indicates that this tissue goes through rhythmical periods of accumulation and utilization as the post-exuvial layers are deposited. This evidence suggests strongly that glycogen is a necessary precursor for chitin formation and is needed not only for this synthesis but for growth of other tissues as well. The fact that glycogen disappears from the inner epidermis on the second day following molt and that little thickening of the inner integument occurs after this period, suggests that the inner integument is completed during a period of three days preceding molt and two days following molt. Large amounts of glycogen, therefore, appear to be utilized at this time by the inner epidermal cells for the synthesis of chitin. That glycogen is a necessary precursor in the synthesis of chitin has also been suggested by Verne (1924, 1926), Renaud (1949), Travis (1951a), Schwabe *et al.* (1952). Renaud (1949) has further proposed a general mechanism by which glycogen may be utilized as a precursor in chitin formation. She suggests that glycogen is first hydrolyzed to glucose, and that this is aminated,

yielding glucosamine which, after acetylation to N-acetyl-glucosamine, undergoes polymerization to yield chitin. During the pre-ecdysial period, hydrolysis of glycogen would contribute greatly to the high blood glucose noted in *Callinectes sapidus* by Baumberger and Dill (1928), in *Astacus fluviatilis* by Damboviceanu (1932), and in *Maia squinado* by Drilhon (1933, 1935). Scheer and Scheer (1951) found that glucose apparently does not serve as a substrate for oxidative metabolism in *Panulirus japonicus* and that it does not stimulate the consumption of O_2 in isolated tissues, since little labeled C^{14} (in injected glucose) was recovered in respiratory CO_2. They further support the idea that the glycogen, present in the hepatopancreas and other tissues, is converted to glucose and that its principal role in the Crustacea is in the formation of chitin. It is interesting that high blood glucose may also be accompanied by increases in organic acids and other intermediates of glycolytic metabolism, thus contributing to the increase in osmotic pressure and facilitating water intake during and following molt. A further, possibly important, function of glycogen during the pre-ecdysial period is an intimate involvement in the deposition of calcospherites in the hepatopancreas. Moog (1946) has suggested the importance of glycogen in calcification of bone. With the abundance of glycogen in the hepatopancreas during Stage D and its possible hydrolysis and subsequent phosphorylation, glycogen could serve indirectly as added substrate (phosphoric esters) for phosphatase action, which, as will be pointed out subsequently, appears to be intimately concerned with the deposition of calcium phosphate calcospherites at the periphery of absorbing cells in the hepatopancreas.

During late Stage D, phosphatase appears in the epidermis and connective tissue. It is localized at the distal ends of the epidermal cells bordering the integument, is present in the reserve cells, and in blood sinuses of the connective tissue. Phosphatase present in these strategic sites of active transfer of materials throughout the integumental tissues, probably participates in those reactions involving hydrolysis and dephosphorylation of glucose phosphate to glucose, which Renaud (1949) suggested to be a probable starting point for chitin formation. Hydrolysis of phosphoric esters would produce molecules which would be able to enter or leave these tissues more readily. The enzyme does not participate in calcification of the branchial integument during the premolt period, since no calcification occurs at this time, although calcium is localized at the distal ends of the epidermal cells of the outer integument and in blood sinuses beneath and among the epidermal cells. In the hepatopancreas alkaline phosphatase is heavily concentrated at the striated borders of absorbing cells. The enzyme is most concentrated around calcium phosphate deposition sites (calcospherites) which are predominantly localized in the distal ends of the absorbing cells. Phosphatase in the hepatopancreas appears not only to be involved in important transfer and dephosphorylation reactions which occur at the surface of the absorbing cells but also to be intimately concerned with the deposition of calcium phosphate calcospherites at the periphery of these same cells. Here the enzyme possibly acts by catalyzing local liberation of phosphate ions from the organic phosphates present in the blood or from those transferred across the cell membranes. Calcium ions which are provided by the blood and which are transferred across the cell membranes then unite with these freed phosphate ions to form deposited calcospherites. It may be pointed out that during this period blood calcium rises from an average intermolt value of 22 mEq/L to a peak premolting average of 41 mEq/L and that total blood phosphorus rises from

3.6 to 5.3 mEq/L. However, little change is observed in blood inorganic phosphate, which remains within the normal intermolt range of 0.28 mEq/L (Travis, 1951a, 1951b). Glycogen, it will be recalled, is abundant in these same tissues and provides a source of phosphoric esters which act as substrate for phosphatase activity. Since the animals do not feed for two weeks preceding and one week following molt, and since the primary source of phosphate is from food (Travis, 1951a, 1951b), all available phosphate must be conserved. The calcospherites represent a means of storing or conserving resorbed phosphate from the old skeleton. Although this will be discussed in a subsequent paper, it may be said that following ecdysis the calcospherites disappear from the hepatopancreas as growth and mineral deposition occurs in the new skeleton.

In addition to the great quantities of glycogen, calcospherites, and heavy localization of phosphatase, the hepatopancreas stores large amounts of lipid during Stages D, A, and B. Other lipid deposits occur around the supra- and sub-esophageal ganglia. It will be recalled that during Stages D, A, and B of the molting cycle, the animals do not feed. It is possible that during such periods of inanition the animals utilize their fat reserves as a major source of energy. This is evident from the work of Renaud (1949) who showed that, although a fraction of protein nitrogen of the hepatopancreas is utilized as an energy reserve during the period of starvation, the reserves that permit *Cancer pagurus* to subsist during this period are constituted mostly from fatty acids and glycerides. Bliss (1952, 1953) has shown that fat and protein appear to be the principal foods oxidized even in normal intermolt *Gecarcinus*, as indicated by the mean respiratory quotient values of 0.77. It would seem, therefore, that during periods of fasting the stores of fat in the hepatopancreas serve as a major source of energy and that they play a principal role in oxidative metabolism of the normal intermolt animal.

The large amounts of fat stored in the hepatopancreas during the pre-ecdysial period may contribute indirectly, through their metabolism, to the rise in blood osmotic pressure preceding molt, which facilitates water intake during and immediately following molt. That fats (fatty acids and glycerols) are present in large quantities within the hepatopancreas and blood preceding molt and that they slowly decline until feeding begins following molt (Stage C) is shown by a number of investigations. Paul and Sharpe (1916) found that prior to molt in *Cancer pagurus, Lithodes,* and *Homarus* there was increased fat storage (fatty acids and glycerides) in the hepatopancreas. Following molt, fatty acids and glycerides decreased in the hepatopancreas and increased in the blood. Damboviceanu (1932) demonstrated a rise in blood fatty acids of *Astacus* before molt, followed by a slow decline during the postmolt period. Drilhon (1935) showed an augmentation before molt of total lipids in the blood, hepatopancreas, and in genital organs of *Maia squinado*. This rise was followed by a fall during and following molt. Renaud (1949) found that total lipids, fatty acids, phosphatides, cholesterol and total unsaponifiable fat increase to a peak in the hepatopancreas preceding molt, slowly fall following molt, and reach their lowest point as the skeleton is hardened (Stage C). Bliss (1952, 1953) reported that eyestalkless *Gecarcinus* approaching molt have an R.Q. greater than one, which to her suggested metabolism of organic acids. It is highly likely that such a situation may occur in normal animals approaching molt. This is very interesting because it would seem from the evidence available that the increase in blood fatty acids, phosphatides, cholesterol and glycerides, as well as organic acids which ac-

cumulate during glycolysis, might very well contribute, as Baumberger and Olmsted (1928) pointed out, to the rise in blood osmotic pressure preceding molt which would facilitate water absorption shortly preceding and following molt.

The fatty acids and cholesterol conveyed to the integumental tissues preceding molt are without doubt used in the formation of the epicuticle. Renaud (1949) showed that in *Cancer pagurus* immediately before formation of the epicuticle, the amount of lipids (fatty acids, cholesterol and unsaponifiable fatty acids) in the epidermis rises from 19% in late Stage C to 33% in Stage D when the epicuticle is being formed. After the epicuticle is formed, the hypodermis is emptied of 1/3 of its lipids.

It is pertinent to suggest the importance of many of these organic acids, products of oxidative and glycolytic metabolism, as carriers of calcium. Such carriers play a part in conveying calcium to the hepatopancreas for storage preceding molt and likewise in conveying it to the integumental tissues as the skeleton progressively calcifies following molt.

It is evident from the foregoing discussion that the marked histological and histochemical changes which occur in the hepatopancreas and integumental tissues during the pre-ecdysial period are associated with the growth of the organism. The hepatopancreas participates intimately in the important processes related to growth of the epidermis. Because it is a major storage depot for glycogen, fat and mineral reserves (calcium phosphate calcospherites) during the pre-ecdysial period, the hepatopancreas is the major organ from which these reserves are mobilized when needed in growth of the integument and other tissues. The possible functions of these reserves in such processes have been suggested.

Summary

1. The integumental tissues from the branchial region of an intermolt spiny lobster (late Stage C) are bordered by an outer and an inner integument. The outer integument consists of four layers: a thin epicuticle, the pigmented layer, the principal layer, and the membranous layer. Both the epicuticle and pigmented layer are formed before molt. The inner integument, about 1/20 the thickness of the outer integument, is composed of two layers, a very thin epicuticle and a uniformly staining endocuticle.

2. The integumental tissues are composed of columnar epidermal cells and a sub-epidermal connective tissue of a loose spongy type. Large oval reserve cells constitute by far the most predominant type of cells in this tissue. These cells store abundant amounts of carbohydrate, lipid and calcium at various stages throughout the molting cycle; they are also found in the hepatopancreas.

3. Within the tubular epithelium of the hepatopancreas are two major types of cells, secretory and absorptive. The former are swollen cells containing very large vacuoles at their distal ends. Secretion is of the apocrine type, leaving only the basal region and nucleus of the cell intact. Reconstitution of secretory cells probably occurs by regeneration from the remaining basal ends. The latter type of cells (absorptive) are tall and cylindrical with a basal or central nucleus.

4. During the intermolt period (late Stage C), a period of active feeding, there is little glycogen, phosphatase, and calcium evident in either the hepatopancreas or the integumental tissues. Lipids, however, are abundant in the epithelial tissues of the hepatopancreas.

5. External signs of an approaching molt (Stage D) become evident by the appearance of a resorptive or ecdysial line along the branchiostegites 3–4 days preceding molting in the summer.

6. The external signs of an approaching molt in *Panulirus* reflect the more basic internal changes which occur in the integumental tissues and hepatopancreas. Growth of the outer epidermis occurs shortly after its retraction from the outer integument, 10–14 days preceding molt. Growth of the epidermis of the inside integument is accomplished through an increase in cell number at 3 days preceding molt. Resorption from the old and completion of the new inner integument occurs in a period of five days, three days preceding and two days following molt. At three days preceding molt the epidermal cells of the outer integument have already resumed an orderly alignment, are greatly elongated, and have secreted the epicuticle and pigmented layer of the new skeleton. By this time most of the principal layer of the old outer integument has been broken down by the molting fluid. The almost complete mineral and organic resorption in some areas, partial in others, allows for complete freeing of the new skeleton from the old and for further thickening of the pre-exuvial layers before ecdysis.

7. During the pre-ecdysial period (late Stage D) large numbers of reserve cells of the integumental tissues become greatly swollen, and bind or store large amounts of polysaccharide material. Glycogen is abundant in the epithelial tissues of the hepatopancreas and simultaneously accumulates in the epidermal cells of the inner integument as the latter is being formed. Available evidence (see discussion) would suggest strongly that glycogen is a necessary precursor for chitin formation. Furthermore, glycogen is perhaps intimately involved in the deposition of calcospherites in the hepatopancreas.

8. During late Stage D, phosphatase appears in the distal ends of the epidermal cells bordering the integument and in reserve cells of the connective tissue. It is suggested that phosphatase in these strategic sites of active transfer probably participates in producing molecules which are able to enter or leave the cells more readily, and in those reactions involving hydrolysis and dephosphorylation of glucose phosphate to glucose, a possible starting point for chitin formation. It is not participating in calcification of the branchial integument at this time, because calcification does not begin until the second day following molt. In the hepatopancreas, on the other hand, alkaline phosphatase is heavily concentrated at the striated border of the absorbing cells and around calcium deposition sites (calcospherites). Here the enzyme appears to be involved in important transfer and dephosphorylation reactions which occur at the surface of the absorbing cells and also to be intimately concerned with the deposition of calcium phosphate at the periphery of these same cells (see discussion).

9. Evidence available suggests that lipids, abundant in the hepatopancreas at this time, function as a major source of energy. Furthermore, the accumulation of fatty acids, glycerides, and other intermediates of oxidative as well as glycolytic metabolism during late Stage D and Stage A may contribute to the rise in osmotic pressure preceding molt, facilitating water intake. Further evidence suggests that some of the fatty acids, cholesterol and unsaponifiable fatty acids conveyed to the integumental tissues preceding molt are used in the formation of the epicuticle.

10. It is suggested that many of the organic acids, products of oxidative and glycolytic metabolism, are perhaps important carriers of calcium. Such carriers

participate in conveying calcium to the hepatopancreas for storage preceding molt, and in conveying it to the integumental tissues as the skeleton progressively calcifies following molt.

LITERATURE CITED

BAUMBERGER, J. P., AND D. B. DILL, 1928. Study of glycogen and sugar content and the osmotic pressure of crabs during the molt cycle. *Physiol. Zool.*, **1**: 545–549.

BAUMBERGER, J. P., AND J. M. D. OLMSTED, 1928. Changes in osmotic pressure and water content of crabs during the molt cycle. *Physiol. Zool.*, **1**: 531–543.

BENSLEY, R. R., AND S. H. BENSLEY, 1938. Handbook of histological and cytochemical technique. Univ. of Chicago Press, Chicago.

BERNARD, C., 1879. Leçons sur les phénomènes de la vie communs aux animaux et aux végétaux., II: 1–564. Baillière, Paris.

BLANK, H., AND P. L. MCCARTHY, 1950. A general method for preparing histologic sections with a water soluble wax. *J. Lab. Clin. Med.*, **36**: 776–781.

BLISS, D. E., 1952. Endocrine control of metabolism in the decapod crustacean, *Gecarcinus lateralis*. Thesis for Ph.D. degree, Radcliffe College.

BLISS, D. E., 1953. Endocrine control of metabolism in the land crab, *Gecarcinus lateralis* (Fréminville). I. Differences in the respiratory metabolism of sinusglandless and eyestalkless crabs. *Biol. Bull.*, **104**: 275–296.

BRONN, H. G., 1896–1907. Klassen und Ordnungen des Tierreichs, **3**: 292–294, 805–806.

CUÉNOT, L., 1893. Études physiologiques sur les crustacés décapods. *Arch. de Biol.*, **13**: 245–303.

DAMBOVICEANU, A., 1932. Composition chimique et physico-chimique du liquide cavitaire chez les crustacés decapods. (Physiologie de la calcification.) *Arch. Roum. Path. Exp. et Microbiol.*, **5**: 239–309.

DENNELL, R., 1947. The occurrence and significance of phenolic hardening in the newly formed cuticle of Crustacea decapods. *Proc. Roy. Soc. London, Ser. B*, **134**: 485–503.

DRACH, P., 1939. Mue et cycle d'intermue chez les crustacés décapods. *Ann. Inst. Oceanogr.*, **19**: 103–391.

DRILHON, A., 1933. La glucose et la mue des crustacés. *C. R. Acad. Sci.*, **196**: 506–508.

DRILHON, A., 1935. Étude biochimique de la mue chez les crustacés. *Ann. Physiol. et Physicochem. Biol.*, **11**: 301–326.

GOMORI, G., 1941. The distribution of phosphatase in normal organs and tissues. *J. Cell. Comp. Physiol.*, **17**: 71–83.

HAWORTH, W. N., 1946. The structure, function and synthesis of polysaccharides. *Proc. Roy. Soc. London, Ser. A*, **186**: 1–19.

HERRICK, F. H., 1895. The American lobster. *Bull. U. S. Fish Comm.*, **15**: 1–252.

HIRSCH, G. C., AND W. JACOBS, 1928. Der Arbeitsrhythmus der Mitteldarmdrüse von *Astacus leptodactylus*. Part I. Der Bewis der Periodizitat. *Zeitschr. vergl. Physiol.*, **8**: 102–144.

HIRSCH, G. C., AND W. JACOBS, 1930. Part II. Wachstum als primarer Faktor des Rhythmus eines polyphasischen organigen Sekretions-system. *Zeitschr. vergl. Physiol.*, **12**: 524–558.

KINCAID, F. D., AND B. T. SCHEER, 1952. Hormonal control of metabolism in crustaceans. IV. Relation of tissue composition of *Hemigrapsis nudus* to intermolt cycle and sinus gland. *Physiol. Zool.*, **25**: 372–386.

KIRCH, J. B., 1886. Das Glycogen in den Geweben des Flusskrebses. Inaug. Diss Bonn. As quoted from Cuénot, 1893.

KRISHNAN, G., 1950. Sinus gland and tyrosinase activity in *Carcinus maenas*. *Nature*, **165**: 364–365.

KRISHNAN, G., 1951. Phenolic tanning and pigmentation of the cuticle in *Carcinus maenas*. *Quart. J. Micr. Sci.*, **92**: 333–342.

KRISHNAN, G., 1954. Tyrosinase activity in relation to phenolic tanning of the cuticle in *Carcinus maenas*. *Proc. Nat. Inst. Sci. India*, **20**: 157–169.

KRUGLER, O. E., AND M. L. BURKNER, 1948. Histochemical observations of alkaline phosphatase in the integument, gastrolith sac, digestive gland and nephridium of the crayfish. *Physiol. Zool.*, **21**: 105–110.

KÜKENTHAL, W., 1926–1927. *Handbuch der Zoologie*, **3**: 848–849, 888–889.
LILLIE, R. D., 1948. Histopathologic technic. The Blakiston Co., Philadelphia.
MANIGAULT, P., 1939. Recherches sur le calcaire chez les mollusques. Phosphatase et précipitation calcique. Histochimie du calcium. *Ann. de Inst. Oceanogr.*, **18**: 331–425.
MEYER, K., 1938. The chemistry and biology of mucopolysaccharides and glycoproteins. *Cold Spring Harbor Symp.*, **6**: 91–101.
MOOG, F., 1946. The physiological significance of the phosphomonesterases. *Biol. Rev.*, **21**: 41–59.
PAUL, J. H., AND J. S. SHARPE, 1916. Studies on Ca metabolism. I. The deposition of lime salts in the integument of decapod Crustacea. *J. Physiol.*, **50**: 183–192.
PEARSE, A. G. E., 1953. Histochemistry theoretical and applied. Little Brown and Company, Boston.
RENAUD, L., 1949. Le cycle des réserves organiques chez les crustacés Décapodes. *Ann. Inst. Oceanogr.*, **24**: 260–357.
RICHARDS, A. G., 1951. The integument of arthropods. Univ. of Minnesota Press, Minneapolis.
RICHARDS, A. G., 1952. Studies on arthropod cuticle. 7. Patent and masked carbohydrate in the epicuticle of insects. *Science*, **115**: 206–208.
RICHARDS, A. G., AND T. F. ANDERSON, 1942. Electron microscope studies of insect cuticle. *J. Morph.*, **71**: 135–183.
ROBERTSON, J. D., 1937. Some features of Ca metabolism of the shore crab (*Carcinus maenas* Pennant). *Proc. Roy. Soc. London, Ser. B*, **124**: 162–182.
SCHEER, B. T., AND M. A. R. SCHEER, 1951. Blood sugar in spiny lobsters. Part I of the hormonal regulation of metabolism in crustaceans. *Physiol. Comp. et Oceol.*, **2**: 198–209.
SCHÖNBORN, E. G. VON, 1910. Beitrage zur Kenntnis Kohlenhydratstoffwechsels bei *Carcinus maenas*. *Zeitschr. f. Biol.*, **55**: 70–82.
SCHÖNBORN, E. G. VON, 1912. Weitere Untersuchungen über den Stoffwechsel der Krustazeen. *Zeitschr. f. Biol.*, **57**: 534–544.
SCHWABE, C. W., B. T. SCHEER AND M. A. R. SCHEER, 1952. The molt cycle in *Panulirus japonicus*. Part II of the hormonal regulation of metabolism in crustaceans. *Physiol. Comp. et Oecol.*, **2**: 310–320.
SCOTT, G. H., 1933. The localization of mineral salts in cells of some mammalian tissues by microincineration. *Amer. J. Anat.*, **53**: 243–287.
STACY, D. M., 1943. Mucopolysaccharides and related substances. *Chem. Ind.*, **62**: 110–112.
TRAVIS, DOROTHY F., 1951a. Calcium metabolism in the decapod Crustacea. Thesis for Ph.D. degree, Radcliffe College.
TRAVIS, DOROTHY F., 1951b. Physiological changes which occur in the blood and urine of *Panulirus argus* Latreille during the molting cycle. *Anat. Rec.*, **111**: 157.
TRAVIS, DOROTHY F., 1951c. Early stages in calcification of the skeleton of *Panulirus argus* Latreille. *Anat. Rec.*, **111**: 124.
TRAVIS, DOROTHY F., 1954. The molting cycle of the spiny lobster, *Panulirus argus* Latreille. I. Molting and growth in laboratory-maintained individuals. *Biol Bull.*, **107**: 433–450.
TRIM, A. R., 1941. Studies in chemistry of the insect cuticle. I. *Biochem. J.*, **35**: 1088–1098.
VERNE, J., 1924. Note histochimique sur le métabolisme du glycogène pendant la mue chez les Crustacés. *C. R. Soc. Biol.*, **90**: 186–188.
VERNE, J., 1926. L'édification de la carapace chitineuse chez les crustacés Décapodes. *C. R. Assoc. Anat.*, **21**: 551–556.
VITZOU, A. N., 1882. Recherches sur la structure et la formation des téguments chez les Crustacés décapodes. *Arch. Zool. Exp. et Gen., Series I*, **10**: 451–576.
WIGGLESWORTH, V. B., 1933. The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* with special reference to the function of the oenocytes and the diurnal glands. *Quart. J. Micr. Sci.*, **76**: 269–318.
WIGGLESWORTH, V. B., 1948. The insect cuticle. *Biol. Rev.*, **23**: 408–451.
YONGE, C. M., 1936. On the nature and permeability of chitin. II. The permeability of uncalcified chitin lining the foregut of *Homarus*. *Proc. Roy. Soc. London, Ser. B*, **120**: 15–41.

ADENOSINETRIPHOSPHATASE ACTIVITY OF SQUID MANTLE MUSCLE (LOLIGO PEALII) [1, 2]

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Studies on the biochemistry of vertebrate striated muscle clearly indicate the presence of two distinct enzymes which catalyze the hydrolysis of the terminal phosphate from adenosinetriphosphate (ATP). One adenosinetriphosphatase (ATPase) is calcium-activated and appears to be identical with myosin (Engelhardt, 1946; Szent-Gyorgyi, 1947). The second ATPase is magnesium-activated and is associated with the particulate matter of the cell (Kielley and Meyerhof, 1948; Perry, 1952; Kitiyakara and Harman, 1953; de Villafranca, 1954). Although it has not been clearly established, most workers assume that myosin-ATPase and the contraction phenomenon must be closely associated. A contribution to the understanding of the enzymes' role, as well as to the general understanding of muscular contraction, could be made by a careful examination of their distribution in muscle tissue from various members of the animal kingdom.

Very few data on muscle ATPase from invertebrates have been published. Humphrey (1949) concluded that there is both a myosin-ATPase and a water-soluble-ATPase in the adductor muscle of the oyster, *Saxostrea commercialis*. More recently Gilmour and Calaby (1952) report two ATPases present in locust muscle (*Locusta migratoria* and *Gastrimargus musicus*); one a calcium-activated, myosin-ATPase and the second, much more concentrated, a magnesium-activated, water-soluble-apyrase. The latter differs from that found in mammalian muscle in not being associated with the particulate matter of the cell.

The large quantity of easily available muscle in the mantle of the squid makes it a highly desirable organism for a comparative study of the biochemistry of muscle. The present paper deals with an attempt to locate and isolate the ATPase activity in the mantle muscle of the squid, *Loligo pealii*. A preliminary account of this work has been published (de Villafranca, 1953).

Materials and Methods

Fresh squid were decapitated and the mantle placed on ice. After removing the fins and pen, the epidermis was stripped from the remainder of the mantle. The mantle was then washed with distilled water and strips, about 5 mm. wide, were cut into 10 volumes of ice cold distilled water and homogenized for one minute in a Waring Blendor run at top speed. The homogenate was centrifuged twice, first at 2300 rpm for 5 minutes and then the supernatant at 4600 rpm (up and

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down) thus separating the homogenate into a water-soluble extract (S_1) and into a residue of debris and water-insoluble material. The combined residues of the two centrifugations (2300 rpm and 4600 rpm) were added to 10 volumes of cold 0.5 *M* KCl to extract the myosin and the KCl homogenate was centrifuged at 2300 rpm for 5 minutes. Precipitation of the myosin by diluting the KCl extract with 6 volumes of cold distilled water was performed twice.

Throughout the extraction the material was kept as cold as possible in ice water baths. Centrifugations were carried out at room temperature. The pH was maintained above neutrality, approximately pH 7.4, by the addition of solid sodium bicarbonate when necessary.

ATPase activity of the various fractions was determined by measuring the increase in inorganic phosphorus after ten minutes incubation at 25° C. of duplicate samples each containing, in addition to the tissue, approximately 0.002 *M* ATP, 0.05 *M* histidine buffer at pH 5.9, 0.05 *M* KCl, and 0.01 *M* $CaCl_2$ or some other ion as specified. The total volume of the reaction mixture was one ml. The reaction was started by the addition of substrate and stopped by trichloroacetic acid (TCA) to a final concentration of 5%. The inorganic phosphorus was determined on the TCA supernate in a Bausch and Lomb colorimeter at 660 mμ according to the method of Fiske and Subbarow (1925), and the enzyme activity expressed as Q_P (Bailey, 1942); *e.g.*, microliter equivalents of phosphorus hydrolyzed in an hour by one mg. of tissue protein.

Protein was estimated by multiplying nitrogen values, obtained by semi-micro Kjeldahl distillation and titration, by the factor 6.25. The tissue proteins were first precipitated with TCA and then digested with a selenium, copper, sulfuric acid mixture before the distillation.

ATP was purchased as the sodium salt (chromatographically pure) from the Schwarz Laboratories, New York City. Inosine-triphosphate (ITP) was a gift from Dr. H. M. Kalckar.

Results

The data from a typical experiment, summarized in Table I, indicate the relative lack of water-soluble ATPase in squid mantle muscle. In this particular experiment the water-soluble fraction (S_1), although containing 18.5% of the mantle protein nitrogen, contains only 0.9% of its ATPase activity. Microscopic examination of this fraction reveals a homogeneous suspension of particles which are, presumably, the mitochondria. Numerous attempts were made to obtain a second ATPase (in addition to a myosin-ATPase) by extracting mantle with molar sucrose, or 0.5 *M* KCl, and following this extraction with differential centrifugation at 20,000 g and 100,000 g to spin down the mitochondria and microsomes, but the results were essentially negative (de Villafranca, 1953). Mitochondrial preparations obtained from sucrose extracts did have somewhat higher proportions of ATPase activity, but these preparations exhibited the same ion activation and pH optimum as the myosin-ATPase. Furthermore, the bulk of the activity could be removed from this type of preparation by washing with 0.5 *M* KCl. The ATPase activity of the soluble fraction (including the particulate matter) was thus considered to be due to myosin-ATPase contamination and not due to an inherent particulate ATPase.

In contrast to the S_1 fraction, the 0.5 *M* KCl extract (MS) of the water-insolu-

TABLE I

The ATPase activity of fractions isolated from squid mantle muscle

Fraction	Q_P	Percentage activity	Percentage nitrogen
Whole homogenate	1140	100.0	100.0
Water-sol. ext. (S_1)	54	0.9	18.5
Residue after KCl ext.	1335	7.6	6.5
KCl extract (MS)	1558	92.3	68.1

ble material contains most of the mantle ATPase activity (Table I). It was from the MS fractions that myosin was precipitated by dilution with 6 volumes of distilled water.

In Figure 1 may be seen the effect of magnesium, manganese, or calcium ions on the ATPase activity of twice-precipitated myosin. Only calcium activates the enzyme to any extent, activating optimally at a concentration of 0.01 *M* $CaCl_2$ both at pH 5.9, as is illustrated, or at pH 8.93. Manganese activates slightly while magnesium is without effect. ATPase activity in the presence of 0.02 *M* $CaCl_2$ was found to be optimal at 0.05 *M* KCl but other concentrations of KCl, up to 0.1 *M*, were only slightly less effective.

The curve illustrating the relationship between myosin-ATPase activity and pH is shown in Figure 2. A definite optimum exists in the region of pH 5.9 with

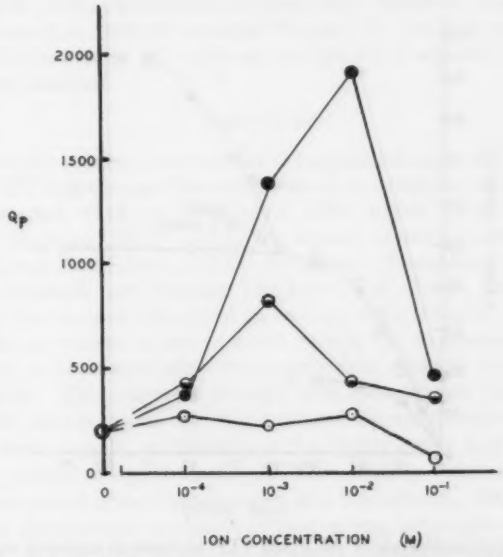

FIGURE 1. The effect of different concentrations of calcium, magnesium, or manganese on the ATPase activity of twice-precipitated squid myosin. Activity was measured in 0.05 *M* histidine buffer (pH 5.9), 0.05 *M* KCl, 0.002 *M* ATP, and the ions as indicated: $CaCl_2$, ●; $MnCl_2$, ◒; and $MgCl_2$, ○.

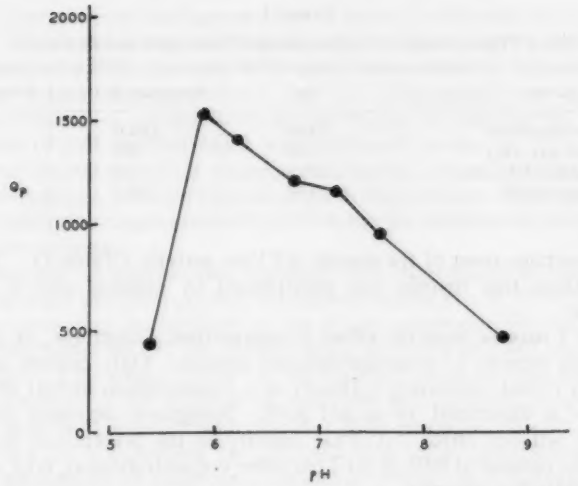

FIGURE 2. ATPase activity of twice-precipitated myosin as a function of pH. Activity measured in 0.05 *M* histidine, 0.02 *M* $CaCl_2$, 0.05 *M* KCl, and 0.002 *M* ATP.

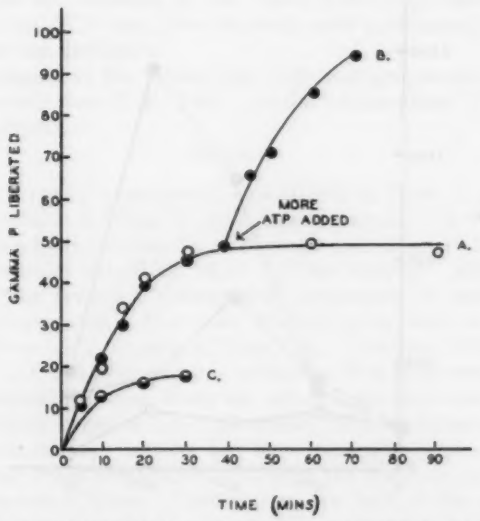

FIGURE 3. Rate of hydrolysis of ATP and ITP by twice-precipitated myosin. In experiment A, 124 gamma of "7 min." P were present; experiment B, 147 gamma and an additional 147 gamma of "7 min." P, aliquot of ATP was added as indicated. Experiment C had ITP, 123 gamma of "7 min." P, as substrate. Activity in all the experiments was measured in 0.05 *M* histidine buffer (pH 5.9), 0.01 *M* $CaCl_2$, and 0.05 *M* KCl.

much greater activity than at pH 8.8 or higher. The most alkaline pH at which the activity was measured was pH 9.4. In this case the activity was proportionally lower than that shown at pH 8.8 (Fig. 2).

That the myosin is probably a true ATPase, splitting only the terminal phosphate from ATP, is indicated in the progress curves (Fig. 3). At the end of 60 minutes approximately 40% of the available "7 min." P had been hydrolyzed (Fig. 3A). If at the end of 40 minutes more substrate is added, additional inorganic phosphorus is released (Fig. 3B), indicating that the enzyme is still active. The enzyme, however, is rapidly inactivated when it is incubated, without substrate, at 37° C. for as little as 10 minutes: 97% of its ATPase activity is lost. It was also noted that much of the activity is lost if the squid are not freshly killed prior to extraction.

Inosinetriphosphate was actively hydrolyzed by squid myosin; Q_P of 1005. This value is not a completely accurate estimate of the ITP hydrolysis since the few experiments performed may reflect a lack of substrate saturation of the enzyme possibly due to high inosinediphosphate content: *i.e.*, at the end of 30 minutes the enzyme preparation had hydrolyzed only 14–16% of the total "7 min." P available (Fig. 3C).

On the assumption that the enzyme hydrolyzed all of the terminal phosphate present in the ATP preparations, the results (in Fig. 3A and of other experiments) indicate that approximately 40% of the "7 min." P is due to the terminal labile group of ATP, or that the substrate preparation contains about 80% ATP. The calculations of the Michaelis-Menten constant were, therefore, based on ATP concentrations calculated as 80% of the total "7 min." P. By this method K_m values, ranging from 2.58 to 3.63×10^{-4} with an average for 5 separate determinations of 3.05×10^{-4}, were obtained.

Discussion

Apparently squid mantle muscle lacks a magnesium-activated, water-soluble or particle-bound, ATPase comparable to that found in vertebrate striated muscle (rat, Kielley and Meyerhof, 1948; de Villafranca, 1954: rabbit, Perry, 1952: or pigeon, Kitiyakara and Harman, 1953) and in the muscle of many invertebrates such as the locust (Gilmour and Calaby, 1952), the oyster (Humphrey, 1949), and, very probably, the cockroach and housefly (Sacktor *et al.*, 1953; Sacktor, 1953). A small amount of this enzyme, too small an amount to be detected under the present conditions, might be present in squid mantle muscle, but its contribution to the total ATPase activity, as compared with the contribution made by myosin-ATPase activity, is negligible. This contrasts strongly with the situation Gilmour and Calaby report for locust muscle where the Mg-activated apyrase dominates the ATPase activity of the whole muscle, particularly in the highly active flight muscles.

The major portion of squid mantle ATPase activity exhibits much the same response to ions as vertebrate (Mommaerts and Seraidarian, 1947; de Villafranca, 1954) and other invertebrate myosin-ATPase; strong activation with calcium (approximately 10^{-2} *M*) and no activation to slight activation with magnesium. In contrast, myosins from the molluscs, *Mya arenaria* (Humphrey, 1948) and *Sepia officinales* (Nguyen-van-Thoai and Pin, 1950), both exhibit greater activity with magnesium than with calcium. It should be remembered, however, that mag-

nesium activation is also a characteristic of undissociated rabbit actomyosin (Szent-Gyorgyi, 1947).

Although the pH optimum for *Loligo* myosin-ATPase is considerably more acid than the optima for mammalian myosin (pH 6.2–6.5 and pH 9.2, Mommaerts and Seraidarian, 1947) it is comparable to the optima reported by Nguyen-van-Thoai and Pin (1950) for other molluscs: pH 5.8 for *Aplysia depilans, Pecten maximus,* and *Sepia officinales.* More alkaline optima were reported for *Mya arenaria* (pH 8.5, Humphrey, 1948) and for *Saxostrea commercialis* (just below pH 8, Humphrey, 1949). Since the unique effect of different buffers on myosin-ATPase activity is well known (Mommaerts and Seraidarian, 1947; Bailey, 1942), it is possible that the acid optimum (pH 5.9) reported here might be a function of the buffer used. Veronal buffer, however, showed no alkaline optimum. Experiments comparing the activity in histidine buffer, glycine buffer and "Tris" buffer at pH 8.8 to 9.0, revealed activity to decrease in that order. The apparent activating effect of histidine is being studied at this time.

It is interesting to note that the specific activity of squid myosin-ATPase is of the same order of magnitude as reported for mammalian myosin; Q_P-s ranging from 1000–6000 (Bailey, 1942). The values here (1000–2000) may be contrasted with the Q_P values reported by Humphrey (1948; 1949) which ranged from 9 to 60 for other invertebrates. The low values reported by Humphrey might be due to inactivation of the enzyme by the high temperatures, 35–38° C., at which it was tested. The squid enzyme, as stated previously, was quickly inactivated at temperatures of that order and the high Q_P values were obtained at 25° C.

Evidently the metabolism of squid muscle deviates somewhat from that of other animals. It would be of more than passing interest to ascertain whether the oxidative enzymes of the tricarboxylic acid cycle, found bound to the particles of mammalian muscle cells, were similarly bound in squid muscle. It would also be of interest to compare the function and chemistry of this muscle, which is composed of smooth muscle fibers (Dahlgren and Kapner, 1908; and the author's own unpublished observations), with striated muscle in an endeavor to clarify the real significance of striations.

Summary

1. It is highly probable that the mantle muscle of the squid, *Loligo pealii,* has only one ATPase, associated with the myosin-like proteins extracted with 0.5 *M* KCl. It lacks a water-soluble, or particulate-bound, ATPase comparable to that found in vertebrate and certain insect muscle.

2. The extremely labile myosin-ATPase is activated almost 10-fold by 0.01 *M* $CaCl_2$ and almost 4-fold by 0.001 *M* $MnCl_2$. Magnesium is without effect. The pH optimum for this enzyme is approximately pH 5.9. A true ATPase, being specific for the terminal phosphate of ATP, it also hydrolyzes ITP at a somewhat reduced rate. An average Michaelis-Menten constant of 3.05×10^{-4} and a Q_P as high as 2000, comparable to mammalian myosin-ATPase and much higher than other marine invertebrates, have been obtained.

LITERATURE CITED

Bailey, K., 1942. Myosin and adenosinetriphosphatase. *Biochem. J.,* **36**: 121–139.

Dahlgren, U., and W. A. Kepner, 1908. A text-book of the principles of animal histology. The Macmillan Co., New York.

ENGELHARDT, V. A., 1946. Adenosinetriphosphatase properties of myosin. *Advances in Enzymol.*, **6**: 147–191.

FISKE, C. H., AND Y. SUBBAROW, 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66**: 375–400.

GILMOUR, D., AND J. H. CALABY, 1952. The magnesium-activated apyrase of insect muscle. *Arch. Biochem. Biophys.*, **41**: 83–103.

HUMPHREY, G. F., 1948. The adenosinetriphosphatase activity of myosins from marine animals. *Physiol. Comp. et Oecol.*, **1**: 89–94.

HUMPHREY, G. F., 1949. Adenosinetriphosphatases in the adductor muscle of *Saxostrea commercialis*. *Physiol. Comp. et Oecol.*, **1**: 266–275.

KIELLEY, W. W., AND O. MEYERHOF, 1948. Studies on adenosinetriphosphatase of muscle. II. A new Mg-activated adenosinetriphosphatase. *J. Biol. Chem.*, **176**: 591–601.

KITIYAKARA, A., AND J. W. HARMAN, 1953. Cytological distribution in pigeon skeletal muscle of enzymes acting on phosphorylated nucleotides. *J. Exp. Med.*, **97**: 553–572.

MOMMAERTS, W. F. H. M., AND K. SERAIDARIAN, 1947. A study of the adenosine triphosphatase activity of myosin and actomyosin. *J. Gen. Physiol.*, **30**: 401–422.

NGUYEN-VAN-THOAI, AND P. PIN, 1950. Sur l'adenylpyrophosphatase de divers Invértébrés marins. *C. R. Acad. Sci., Paris*, **231**: 1580–1582.

PERRY, S. V., 1952. The adenosinetriphosphatase activity of lipoprotein granules isolated from skeletal muscle. *Biochim. et Biophys. Acta*, **8**: 499–509.

SACKTOR, B., 1953. Investigations on the mitochondria of the house fly, *Musca domestica* L. I. Adenosinetriphosphatases. *J. Gen. Physiol.*, **36**: 371–387.

SACKTOR, B., G. M. THOMAS, J. C. MOSER AND D. L. BLOCH, 1953. Dephosphorylation of adenosine triphosphate by tissues of the American cockroach, *Periplaneta americana* (L). *Biol. Bull.*, **105**: 166–173.

SZENT-GYORGYI, A., 1947. Chemistry of muscular contraction. Academic Press, Inc., New York.

DE VILLAFRANCA, G. W., 1953. The apyrase activity of mantle muscle of *Loligo pealii*. *Biol. Bull.*, **105**: 392–393.

DE VILLAFRANCA, G. W., 1954. Adenosinetriphosphatase activity in developing rat muscle. *J. Exp. Zool.* (in press).